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UNIVERSITY OF ALBERTA

PERTUSSIS TOXIN BINDING TO CERULOPLASMIN

BY

ROBERT ALEXANDER BONNAH

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FUFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

DEPARTMENT OF

MEDICAL MICROBIOLOGY AND INFECTIOUS DISEASES

EDMONTON, ALBERTA

SPRING, 1991

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DEDICATION

This thesis was written in loving memory of Bessie Alma Holliday.

ABSTRACT

Using the "western" blotting procedure, I have identified glycopeptides in ceruloplasmin capable of binding to ^{125}I-labeled pertussis toxin (^{125}I-PT). In this system, iodinated PT bound to ceruloplasmin peptides with molecular weights of 138K, 122K, 72K, and with reduced avidity to 79K and 50K fragments. Binding was inhibited by unlabeled PT, which demonstrated the utility of PT, radio-iodinated in the presence of fetuin-agarose, as a probe for receptors on western blots. Using the fetuin-coated polystyrene tube inhibition assay, it was shown that reduction and alkylation had no apparent effect on the PT-binding activity of ceruloplasmin glycopeptides. However, limited enzymatic cleavage or the specific removal of *N*-linked oligosaccharides from ceruloplasmin dramatically decreased its PT-binding activity in the western blot and polystyrene tube assay.

Attempts to purify ceruloplasmin peptides with PT-binding activity by affinity chromatography were unsuccessful. Therefore, it was necessary to isolate individual ceruloplasmin fragments from SDS-polyacrylamide gels. Western blotting experiments using the isolated ceruloplasmin peptides demonstrated that the 72K (67K) fragment of ceruloplasmin contains all of the structural components required for optimal PT binding. However, PT binding to the 50K peptide was not observed with the western blot assay. In contrast, in the polystyrene tube assay, the 50K fragment displayed strong PT-binding activity. Both the

72K (67K) and 50K fragments were subsequently shown to aggregate in solution. These findings are consistent with the hypothesis that PT prefers binding to receptors that provide an opportunity for multiple glycan attachment sites. On western blots, the single *N*-linked glycan on the 50K peptide may not be able to assume a configuration that satisfies this criterion. In the solution phase, however, there may be fewer constraints on the spatial orientation of the oligosaccharides in the aggregated form of the 50K peptide. The 50K aggregates may, therefore, present PT with a surface containing multiple glycan attachment sites. In summary, the results of my investigations support the hypothesis that binding of PT to glycopeptides is modulated by the number of oligosaccharide units attached to the peptide backbone. In addition, the functional importance of *N*-linked oligosaccharide chains was confirmed.

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LIST OF SYMBOLS AND ABBREVIATIONS

ACT	adenylate cyclase toxin
B. pertussis	*Bordetella pertussis*
CHO	Chinese hamster ovary
DNS-Cl	Dansyl chloride
DNT	dermonecrotic toxin
FHA	filamentous hemagglutinin
Gal	galactose
GlcNAc	*N*-Acetylglucosamine
HAI	hemagglutination inhibition
HPLC	high performance liquid chromatography
MBq	MegaBecquerel
$Na^{125}I$	radioisotope 125 Iodine (Sodium salt)
N-linked	asparagine-linked
NeuAc	*N*-Acetylneuraminic acid
O-linked	Serine or Threnonine-linked
PBS	sodium phosphate buffered saline
PT	pertussis toxin
R/A	reduced and alkylated
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
TCT	tracheal cytotoxin
WGA	wheat germ agglutinin

1.0 INTRODUCTION

Whooping cough is a severe disease of humans caused by an upper respiratory tract infection by the bacterium *Bordetella pertussis* (Cherry, J.D., *et al.*, 1988). Whooping cough is a disease of both children and adults, though it is typically more severe in children and adolescents (Cherry, J.D., *et al.*, 1988). In fact, the majority of deaths from pertussis infections occur in children under the age of one year (Nelson, J.D., 1978). It is likely that these children are not protected from disease by immunization because their immune systems are underdeveloped (Cherry, J.D., *et al.*, 1988). Transmission of the disease occurs primarily by the inhalation of aerosols produced during the coughing episodes of infected individuals (Cherry, J.D., *et al.*, 1988). After inhalation, the organism uses a variety of adhesins to specifically attach to ciliated epithelial cells of the upper respiratory tract; where it remains localized. Once established, the organism begins to multiply and produce various virulence factors, some of which are disseminated throughout the host (Weiss, A. A., and Hewlett, E.L., 1986). These virulence factors and biologically active components may individually or collectively produce serious disease symptoms (Munoz, J.J., 1985).

The clinical disease of whooping cough in humans is characterized by three phases; a catarrhal phase, a

paroxysmal phase, and convalescence (Conner, J.D., 1981). The catarrhal phase usually occurs within five to seven days post-infection, and is typified by cold-like symptoms. At this point, although the organism can easily be isolated from nasopharyngeal swabs, the disease usually goes undiagnosed unless pertussis is suspected through prior contact with an infected individual.

Following the catarrhal phase, the more serious symptoms of the paroxysmal phase begins. This phase is characterized by a dry, non-productive cough that evolves into the characteristic paroxysmal cough in which large amounts of mucous are produced. The coughing episodes typically last thirty seconds, during which time the patient has little chance to inhale. At the end of the violent coughing episode, the patient gasps for air and produces a characteristic "whoop" sound, from which the name of the disease was derived. Other pathophysiologic symptoms of a pertussis infection include a pronounced lymphocytosis, hypoglycemia, weight loss and occasionally in children, encephalopathy.

The paroxysmal stage typically lasts 30-40 days, however it can last for periods of six months before convalescence. Due to the distinctive clinical signs of the paroxysmal phase, a rapid diagnosis is possible. However, the majority of the clinical observations during the paroxysmal phase are due to the systemic spread of toxins produced by the organism (Weiss, A.A., and Hewlett,

E.L., 1986). Thus, although *B. pertussis* may be eliminated from the host using antibiotics, an individual may display the clinical symptoms of pertussis for a significant period of time after the organisms can no longer be isolated.

There are a variety of virulence factors produced by *B. pertussis*, including: agglutinogens, filamentous hemagglutinin (FHA), adenylate cyclase toxin (ACT), dermonecrotic toxin (DNT), tracheal cytotoxin (TCT), lipopolysaccharide (endotoxin), and pertussis toxin or PT (Weiss, A.A., and Hewlett, E.L., 1986). Pertussis toxin (PT), is a major virulence factor produced by *B. pertussis* and is also an important protective antigen in the killed whole cell pertussis vaccine preparations currently used in North America and Europe (Munoz, J.J., *et al.*, 1981; Sato, H. and Sato, Y., 1984). PT exhibits an array of biological effects, including the ability to induce lymphocytosis, alter the host immune system, and disrupt glucose homeostasis (Morse S.I., and Morse, J.H., 1976; Yajima, M., *et al.*, 1978; Meade, B.D., *et al.*, 1984; Munoz, J.J., 1985). PT can be excreted from the organism or remain associated with the cell surface. In its cell-associated form, PT may be partially involved in attachment of the organism to ciliated epithelial cells (Tuomanen, E., and Weiss, A.A., 1985).

Pertussis toxin has a classical A-B structure (Gill, D.M., 1978) in which the "A" or active component displays enzymatic activity, and the "B" or binding constituents

facilitate attachment of the toxin to host cell receptors. The B subunit also provides a mechanism for penetration of the active component through the cytoplasmic membrane (Tamura, M. *et al.*, 1982; Tamura, M., *et al.*, 1983; see figure 1 for an illustration of PT). The toxin is composed of five dissimilar polypeptides, designated S1-S5 (Tamura, M., *et al.*, 1982; Peppler, M.S., *et al.*, 1985), though a high degree of homology (67-70%) exists between the S2 and S3 subunits as predicted by the nucleotide sequences of the respective genes (Locht, C., and Keith, J.M., 1986; Nicosia, A., *et al.*, 1986). The largest subunit, S1 ("A" component), mediates adenosine diphosphate (ADP)-ribosylation of the α component of a group of homologous guanine-nucleotide dependent regulatory complexes commonly known as G or N proteins (Gilman, A.G., 1987) which are involved in hormonal signal transduction. The S2-S5 subunits can interact to form a pentameric base ("B" component) that facilitates PT binding to host cell receptors (Tamura, M., *et al.*, 1983). It is believed that two dimers are formed by the interaction of S2 and S4 (dimer one) and S3 and S4 (dimer two), the two dimers being joined by the S5 subunit to form the pentameric base (Tamura, M., *et al.*, 1982). Earlier studies demonstrated the ability of both dimer one and dimer two to inhibit the biological activity of PT in rat adipocytes (Tamura, M., *et al.*, 1983) and to inhibit the agglutination of erythrocytes

Figure 1. Schematic Representation Of PT (Top View). PT represents an "A-B" toxin where the "A" component mediates an enzymatic function and the "B" or binding constituents facilitate binding to host cell receptors. Molecular weight of the holotoxin is 104,950*.

S1 - The S1 subunit of PT ("A" or active component). This subunit mediates the toxin's enzymatic function; the adenosine diphosphate (ADP)-ribosylation of host G proteins. The molecular weight of this subunit is 26,024*.

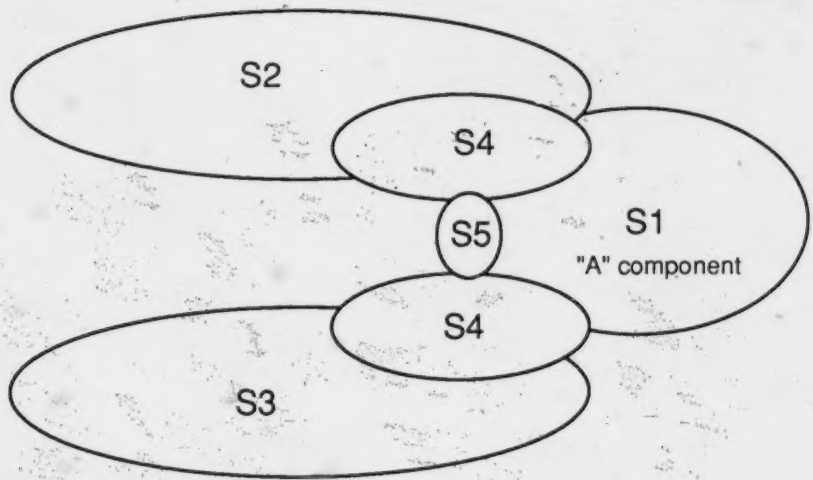
S2 - The S2 subunit of PT (part of the "B" oligomer). This subunit forms a dimer with one of the S4 subunits (dimer one). The molecular weight of this subunit is 21,924*.

S3 - The S3 subunit of PT (part of the "B" oligomer). This subunit forms a dimer with the other S4 subunit (dimer two). The molecular weight of this subunit is 21,873*.

S4 - The S4 subunits of PT (part of the "B" oligomer). There are two S4 subunits in PT; one subunit interacts with the S2 subunit and the other interacts with the S3 subunit. The molecular weight of this subunit is 12,058*.

S5 - The S5 subunit of PT (part of the "B" oligomer). This subunit is responsible for joining dimer one and dimer two together. The molecular weight of this subunit is 11,013*.

*Based on the nucleotide sequence analysis (Locht, C., and Keith, J.M., 1986).


S2
S4
S5
S1
"A" component
S4
S3

(Nogimori, K., *et al.*, 1984). Thus, the eukaryotic receptor binding site on the B oligomer may be common to both dimers.

There are a variety of practical *in vitro* models which have been used to examine the activities of PT on different cell types, some of which are sensitive to nanomolar concentrations of PT. First, PT induces a clustered growth pattern in Chinese hamster ovary (CHO) cell monolayers (Hewlett, E.L., *et al.*, 1983). A second model is the PT-mediated agglutination of untreated (Irons, L.I., and Maclennan, A.P., 1979) or chymotrypsin-treated (Armstrong, G.D., and Peppler, M.S., 1987) goose erythrocytes. PT also causes the release of glycerol from rat epididymal adipocytes (Endoh, M., and Nakase, Y., 1982). Assays for PT based on ADP-ribosyltransferase and NAD-glycohydrolase activities have also been described (Moss, J., and Vaughan, M., 1984; Ribeiro-Neto, F., *et al.*, 1985). At the molecular level, two mechanisms of pathogenesis are proposed for PT. First, inactivation of normal eukaryotic cell metabolism occurs concomitantly with the ADP-ribosylation of host G proteins (Gilman, A.G., 1987). Second, the lectin-like binding of the B oligomer to host cell receptors is believed to act mitogenically, causing lymphocytosis and other immunomodulating activity (Munoz, J.J., 1985).

Further analysis of the interactions of PT with CHO cells has yielded other important findings. A recent study

implies that the receptor for PT on CHO cells is a 165K glycoprotein (Brennan, M.M., *et al.*, 1988). The results from this study demonstrated that a variant CHO cell line (clone 15B) which lacked the terminal sialyllactosamine NeuAcßGal(1-4)GlcNAc residues on asparagine- or *N*-linked oligosaccharides, was resistant to toxin activity (Brennan, M.M., *et al.*, 1988). Furthermore, in addition to interacting with eukaryotic cells such as goose erythrocytes and CHO cells, PT is known to have lectin-like activity, binding strongly to a variety of glycosylated proteins. Several studies have examined the interactions of PT with sialoglycoproteins such as fetuin, haptoglobin, transferrin, glycophorin A, α1 acid glycoprotein, fibronectin, and ceruloplasmin (Capiau, C., *et al.*, 1986; Witvliet, M.H., *et al.*, 1989; Tyrrell, G.J., *et al.*, 1989; Heerze, L.D., and Armstrong, G.D., 1990). A study examining the interaction of PT with fetuin, a bovine serum sialoglycoprotein, demonstrated that the binding of PT to asialoagalacto-[*N*-acetylglucosamino]fetuin was dramatically reduced in comparison to that of native fetuin, and that full binding activity could be restored by the sequential addition of specific carbohydrate groups (Armstrong, G.D. *et al.*, 1988). In comparison to the Brennan (1988) study, this study provided more direct evidence that the carbohydrate moiety of glycoproteins was conferring PT-binding activity.

In addition to the terminal carbohydrate sequence of αNeuAc(2-6)ßGal(1-4)GlcNAc, many other important structural characteristics of the oligosaccharide chains of glycoproteins that are important for PT-binding activity were demonstrated in other studies. Initial studies by Sekura *et al.*, (1985) suggested that PT only interacted with asparagine or *N*-linked oligosaccharide chains, and oligosaccharides linked to serine or threonine residues (*O*-linked) were not involved in PT binding activity. In addition, a study by Armstrong, *et al.*, (1988) contributed further evidence that only *N*-linked oligosaccharide chains were involved in PT-binding to glycoproteins. However, although many characteristics of PT binding to glycoproteins were discovered from these earlier studies, the relationship between PT receptors on fetuin (and other glycoproteins), and receptors found on erythrocytes and host tissue cells has not clearly been established.

To further examine the lectin-like binding activity of the B oligomer of PT, a direct comparison of PT to wheat germ agglutinin (WGA), a plant lectin, was made (Armstrong, G.D., *et al.*, 1988). WGA is a protein with a molecular weight of 36,000 and is composed of two similar polypeptide chains, each possessing two binding sites (Nagata, Y. and Burger, M.M., 1974). Prior studies in which the interaction of WGA with various sialoglycoproteins was examined, demonstrated that several factors were involved in lectin-glycoprotein binding. First, the importance of

valency for optimal binding activity was assessed (Bhavanandan, V.P., and Katlic, A.W., 1978). The results of these investigations confirmed that optimal binding occurred when the density of the oligosaccharide units was such that saturation of the binding sites could be achieved with minimal steric hindrance. Other WGA studies have also demonstrated that the lectin apparently prefers binding to terminal α(2-3)-linked *N*-Acetylneuraminic acid (NeuAc) residues suggesting that lectin-carbohydrate interactions are stereospecific (Kronis, K.A., and Carver, J.P., 1985; Furukawa, K.A., *et al.*, 1986). However, the stringency of this interaction is not absolute in that sialoglycoproteins with terminal α(2-6)-linked NeuAc residues are also capable of interacting with WGA (Kronis, K.A., and Carver, J.P., 1985; Furukawa, K.A., *et al.*, 1986). PT, however, may prefer binding to oligosaccharides terminating in α(2-6)-linked NeuAc residues (Armstrong, G.D., *et al.*, 1988).

Based on the PT-WGA analogy and other studies demonstrating PT's lectin-like properties (Brennan, M.M., *et al.*, 1988), a study by Tyrrell *et al.*, (1989) examined the interactions of PT and WGA with several sialoglycoproteins including bovine fetuin and the human glycoproteins α1-acid glycoprotein, glycophorin A, haptoglobin and transferrin. In this study, a polystyrene tube inhibition assay was used to determine the ability of each of the glycoproteins to inhibit the binding of PT or WGA to fetuin. The results conclusively demonstrated

differential binding of the PT and WGA to the glycoproteins, implying that PT and WGA molecules can differentiate between subsets of receptors bearing similar sugar sequences. It was proposed that the differential binding may reflect dissimilarities between the structures of PT and WGA, and the unique geometrical orientation of the oligosaccharide units on the glycoproteins. It was also suggested that the differences in binding may be due to the variable content of terminal α(2-3) and α(2-6)-linked NeuAc residues on the various glycoproteins.

In an extension of the PT-WGA analogy, a recent study by Heerze and Armstrong (1990) compared the lectin-like activity of PT with two plant lectins that have differential specificities for α(2-6) and α(2-3)-linked NeuAc. A hemagglutinin known as SNA from elderberry bark (*Sambucus nigra*), which has a binding specificity for terminal NeuAcα(2-6)Gal sequences (Shibuya, N., *et al.*, 1987), was found to bind a variety of glycopeptides with specificity similar to PT. The binding of PT and SNA was different from that of the leukoagglutinin from the seeds of *Maackia amurensis* (MAL) which preferentially binds terminal NeuAcα(2-3)Gal sequences (Wang, W-C., and Cummings, R.D., 1988). This study further supported theories that lectin-carbohydrate interactions are stereospecific and that PT preferentially binds to terminal NeuAcα(2-6)Gal sequences. The similar but not identical binding affinities of SNA and PT to the glycopeptides

provided yet additional evidence that this property is a general characteristic of lectins.

Although many of the structural features of the carbohydrates of glycoproteins had been investigated, it was apparent that PT receptor activity of glycoproteins may be modulated by factors other than the sequence and structure of the oligosaccharide units. First, it was demonstrated that much higher concentrations of neuraminlactose than fetuin were required to inhibit the PT-mediated agglutination of goose erythrocytes. Second, it was found that glycopeptides derived from fetuin were unable to inhibit the hemagglutination reaction (Armstrong, G.D., *et al.*, 1988). It was suggested that the number of antennae in branched oligosaccharide units, the number (valency) of oligosaccharide units on glycoprotein receptors, or structural components of the core sugars or peptide sequences may also contribute to binding activity. Thus, like WGA, PT interactions with glycoproteins are likely dependent on the correct spatial orientation of the specific carbohydrate residues. It is speculated that multivalent interactions are responsible for the high avidity demonstrated (Ochoa, J.L., 1981).

Although the sequence and linkage of the specific carbohydrate residues of *N*-linked oligosaccharide chains required for optimal PT binding had been determined, additional structural features had not been evaluated until recently. More specifically, it was unknown if a

biantennary oligosaccharide structure was sufficient for high affinity PT interactions, or if a triantennary or tetraantennary oligosaccharide was required. It has been recently demonstrated that fibrinogen, which contains solely biantennary *N*-linked oligosaccharide chains, binds to PT in a modified fetuin-coated polystyrene tube inhibition assay with a higher avidity than fetuin (Heerze, L.D., and Armstrong, G.D., 1990), which has a mixture of biantennary and triantennary oligosaccharide structures (Green, E.D., *et al.*, 1988; Cumming, D.A., *et al.*, 1989). It is apparent, however, that PT is capable of interacting with triantennary and tetraantennary oligosaccharide structures (Tyrrell, G.J., *et al.*, 1989; Heerze, L.D., and Armstrong, G.D., 1990). The avidity of these interactions was found to vary.

The number (valency) of *N*-linked oligosaccharide chains required for optimal PT binding is currently unknown. Previous studies have demonstrated that PT binds to human glycophorin A (Tyrrell, G.J., *et al.*, 1989; Witvliet, M.H., *et al.*, 1989), and human transferrin (Tyrrell, G.J., *et al.*, 1989). Though both reports suggested that glycophorin is capable of binding to PT, the avidity of the interaction was found to be quite different. However, different assays were used in the two publications, and this may provide insight as to the reason for this apparent discrepancy (Tyrrell, G.J., *et al.*, 1989; Witvliet, M.H., *et al.*, 1989).

Glycophorin A contains fifteen *O*-linked oligosaccharide units, but only one (biantennary) *N*-linked oligosaccharide chain (Tomita, M., *et al.*, 1978). The sequence and structure of the *N*-linked sugars of Glycophorin A have been determined, and the proposed models suggest the oligosaccharide chain has a terminal carbohydrate sequence of αNeuAc(2-6)ßGal(1-4)GlcNAc. When examining the PT-glycophorin interaction using the polystyrene tube inhibition assay, a high avidity interaction was seen (Tyrrell, G.J., *et al.*, 1989). However, when PT-glycophorin binding was analyzed using a dot blot assay with glycophorin attached to a solid matrix (nitrocellulose), then incubated with aqueous PT, a low avidity interaction was seen (Witvliet, M.H., *et al.*, 1989). A possible explanation for this discrepancy is that the effective valency (number of *N*-linked oligosaccharide chains) may vary in the two assays. In the polystyrene tube assay, where the inhibiting proteins are in solution, it is possible that glycophorin molecules aggregate and thus the effective valency is greater than one. This is quite likely, as it is well established that glycophorin A exists as large aggregates in solution (Furthmayr, H., *et al.*, 1975). However, on a solid matrix such as nitrocellulose, glycophorin may behave differently, and the wrong spatial orientation of the oligosaccharide chains may occur. Consequently, on the nitrocellulose, the effective valency may be less than optimal. It should be noted that

excessive aggregation of glycophorin is unlikely to occur *in vivo*, as glycophorin is an integral membrane protein of erythrocytes (Furthmayr, H., *et al.*, 1975). Due to the conflicting reports, and the fact that glycophorin aggregates in solution, I was not confident that the true valency of glycophorin (one *N*-linked oligosaccharide chain) was representative of the actual valency that occurred when glycophorin was in solution or on a solid matrix. Furthermore, I anticipated the use of a western blot type procedure to examine PT-glycoprotein interactions. The low avidity of PT-glycophorin interactions in the dot blot assay (Witvliet, M.H., *et al.*, 1989) where glycophorin is attached to a solid matrix, suggested that the western blot assay would be of limited applicability. For these reasons, glycophorin was not chosen as a model glycoprotein to examine valency.

Human transferrin contains two (primarily) biantennary *N*-linked oligosaccharides with terminal αNeuAc(2-6)ßGal(1-4)GlcNAc (Spik, G., *et al.*, 1975; Petrén, S., and Vesterberg, O., 1989). Transferrin has PT-binding activity, however the avidity of the interaction is lower than that of fetuin (Tyrrell, G.J., *et al.*, 1989; Heerze, L.D., and Armstrong, G.D., 1990). This lower avidity may be due to the geometrical orientation of the oligosaccharide chains, and/or may also be due to PT requiring a valency of greater than two oligosaccharide chains for optimal PT binding. In addition to low PT

binding activity, there is a large degree of structural heterogeneity of the carbohydrate moieties of transferrin (März, L., *et al.*, 1982; Petrén, S., and Vesterberg, O., 1989). These factors do not allow for its use as a model glycoprotein to characterize receptors for PT.

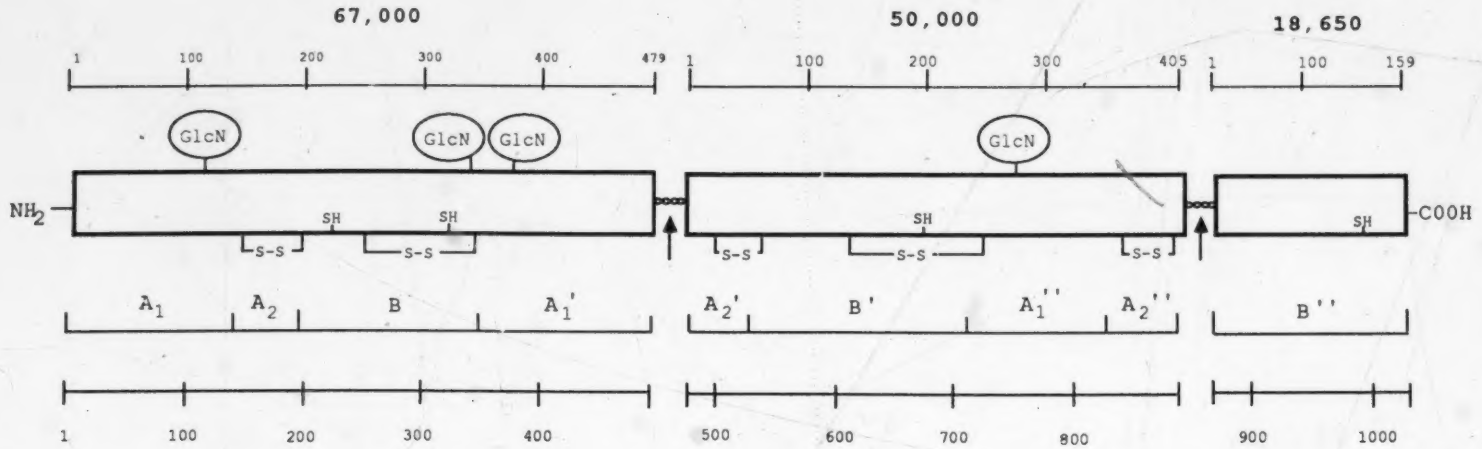
I wanted to further examine additional structural components of the core sugars or peptide sequences of glycoproteins that may also contribute to PT binding activity. Though at the onset of my investigations, a wealth of knowledge was available about the complex oligosaccharide structures of fetuin (Green, E.D., 1988; Cumming, D.A., *et al.*, 1989) the complete amino acid sequence and attachment sites for the oligosaccharide units on the fetuin molecule had not yet been delineated. Thus, investigation of the structural components of the peptide sequences which may also contribute to PT-binding activity using fetuin as a model were limited [though the complete cDNA and amino acid sequence of fetuin have since been determined (Dziegielewska, K.M., *et al.*, 1990)]. Further, microheterogeneity within the population of homologous *N*-linked oligosaccharide units on the fetuin molecule also limited the utility of fetuin as a model glycoprotein (Green, E.D., *et al.*, 1988; Cumming, D.A. *et al.*, 1989).

Another glycoprotein, ceruloplasmin, with four *N*-linked oligosaccharide chains, was believed to be of use to examine some of the additional structural features that contribute to the stability of the lectin-glycoprotein

interaction. Ceruloplasmin, a 132K copper binding protein synthesized in the liver, is another example of a serum glycoprotein with PT binding activity. As a plasma protein, ceruloplasmin possesses several primary functions. First, it represents the major copper carrier in plasma and is aqua-blue in color when isolated in its copper-carrying form at high concentration. It is suggested that there are 6 or possibly 7 copper atoms bound to every ceruloplasmin molecule (Magdoff-Fairchild, B., *et al.*, 1969). Second, it is responsible for the ferroxidase activity in plasma. Last, it is a scavenger of free radicals and superoxide ions (Arnaud, P. *et al.*, 1988).

The native ceruloplasmin molecule is produced as a 132K precursor, consisting of three proteolytic domains with molecular weights of 67K (referred to as the 72K fragment in this thesis because of its mobility on SDS-PAGE gels), 50K and 19K (see figure 2; Ortel, T.L., *et al.*, 1983; Ortel, T.L., *et al.*, 1984). The precise linkage sites of four *N*-linked oligosaccharides, analogous to those found in fetuin, are also known. Three of the four *N*-linked oligosaccharides are present in the 72K (67K) domain, while the fourth resides in the 50K fragment, and the 19K fragment remains unglycosylated. The native molecule has a strong tendency to undergo autoproteolysis following synthesis and during purification procedures (Ortel, T.L., *et al.*, 1984). Consequently, when analyzed by SDS-PAGE, several major bands and minor bands are seen.

Figure 2. Structural Model Of Human Ceruloplasmin Based On Sequence Analysis (Takahashi, N., *et al.*, 1984), Internal Homology, Hydrophilic/Hydrophobic Character, Secondary Structure, And Tryptic Cleavage Sites (Takahashi, N., *et al.*, 1983; Ortel, T.L., *et al.*, 1984). The three fragments (molecular weights of 67,000, 50,000, and 18,650) are aligned in the proposed order. Probable disulfide bonds, (-S-S-) and the locations of the *N*-linked oligosaccharides (GlcN), amino- (NH_2) and carboxyl- (COOH) terminal residues are displayed. The location of the three major tryptic cleavage sites are indicated by arrows. The model also indicates the proposed series of two (A,B) or three (A_1, A_2, B) sets of repeating homologous domains.

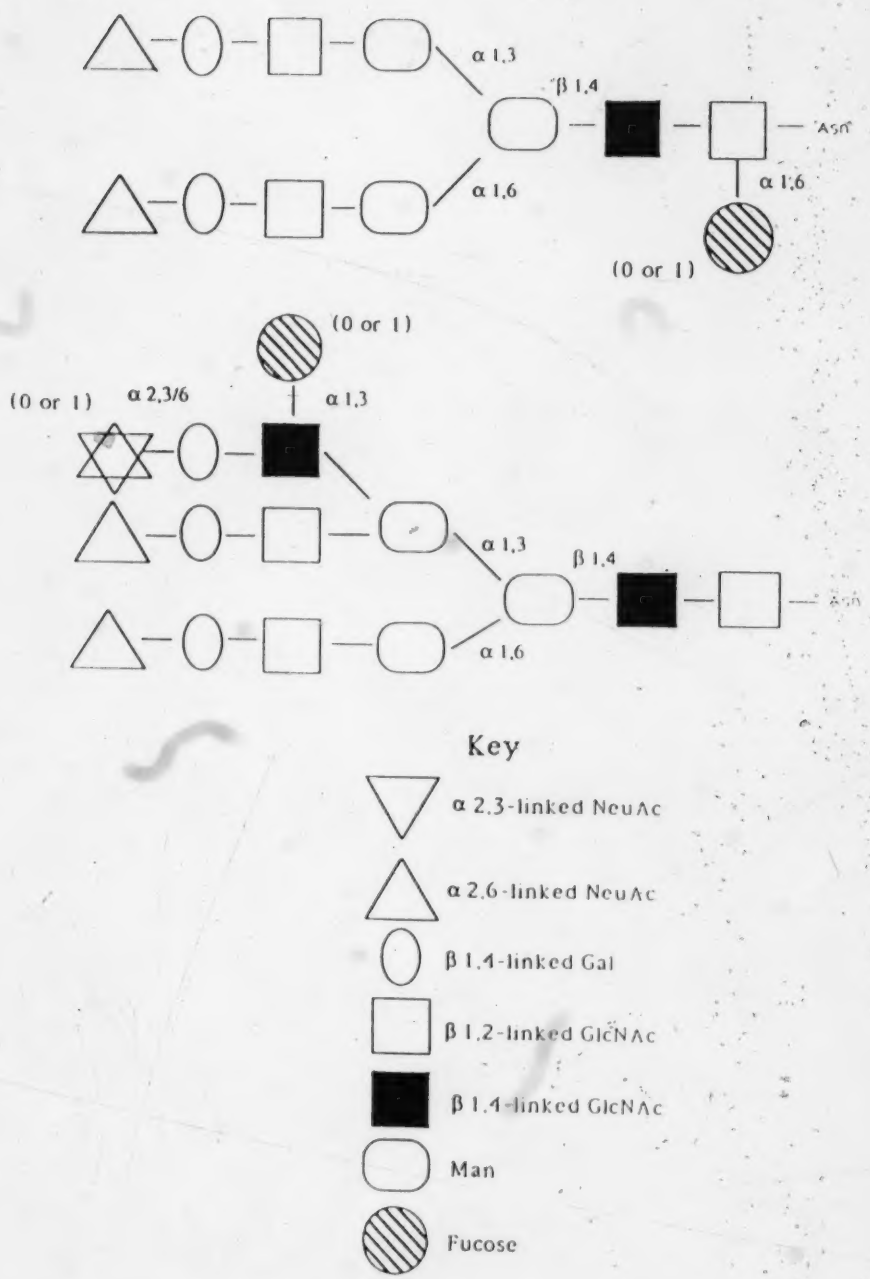


Scale: number of residues

In contrast to fetuin, the complete amino acid sequence and oligosaccharide attachment sites of ceruloplasmin have been known for some time (Takahashi, N., *et al.*, 1984). The amino acid sequence analysis has revealed internal triplication in the primary structure of the entire molecule. It has been suggested that the molecule evolved by tandem triplication of ancestral genes. Consequently, current models suggest an alternating structure of six domains of two different kinds (figure 2; Takahashi, H., *et al.*, 1983; Ortel, T.L., *et al.*, 1984). Furthermore, structural and sequence data of the biantennary and triantennary oligosaccharide units of ceruloplasmin (Yamashita, K. *et al.*, 1981) and the microheterogeneity of the complex structures (Endo M., *et al.*, 1982) are also known (see figure 3).

Ceruloplasmin represented an attractive glycoprotein for studying the valency required for optimal PT binding activity, due to the three *N*-linked oligosaccharide chains on the 67K fragment (72K fragment), and one on the 50K fragment (Takahashi, N., *et al.*, 1984). Because of the autoproteolytic nature of ceruloplasmin, the 72K (67K) fragment can be isolated from the native molecule. I reasoned that by comparing the PT binding activity of native ceruloplasmin (four *N*-linked oligosaccharides) relative to other individual ceruloplasmin fragments, additional information about the valency required for optimal PT binding could be obtained. If the 72K (67K)

Figure 3. Structures Of The Biantennary And Triantennary Oligosaccharide Chains Of Human Ceruloplasmin (adapted from Yamashita, K., *et al.*, 1981; Endo, M., *et al.*, 1982).


α 1,3
β 1,4
Asn
α 1,6
α 1,6
(0 or 1)
(0 or 1)
(0 or 1)
α 2,3/6
α 1,3
α 1,3
β 1,4
α 1,6
Key
α 2,3-linked NeuAc
α 2,6-linked NeuAc
β 1,4-linked Gal
β 1,2-linked GlcNAc
β 1,4-linked GlcNAc
Man
Fucose

fragment were able to inhibit the binding of PT as well as native ceruloplasmin, it would be possible to speculate that optimal binding of PT to glycoproteins requires no more than three oligosaccharides. If the 50K ceruloplasmin fragment bound to PT only with a low affinity, it would be reasonable to speculate that a valency of one oligosaccharide chain was insufficient for optimal PT-binding activity. In addition to the academic interest, this information could be useful in the development of novel diagnostic or therapeutic reagents for detecting or treating whooping cough. These reagents might be based on synthetic receptor analogs that could be used to specifically absorb PT from clinical samples, or neutralize toxin activity in the acutely ill patient.

The objective of this study was to examine the lectin-like properties of pertussis toxin through its interactions with ceruloplasmin and ceruloplasmin peptides, and to attempt to identify the minimal structure of ceruloplasmin necessary for interaction with PT. By using the fetuin-coated polystyrene tube inhibition assay (Tyrrell, G.J., *et al.*, 1989) and the western blotting procedure (Burnette, W.N., 1981), information concerning the importance of valency, the peptide backbone, and the importance of the oligosaccharide chains of ceruloplasmin could be obtained.

2.0 EXPERIMENTAL PROCEDURES

2.1 Materials:

Human ceruloplasmin and pertussis toxin from *Bordetella pertussis* were donated by the Connaught Institute for Biotechnology Research, Willowdale, Ontario. Polystyrene tubes were purchased from Fisher Scientific, Canada. Fetuin-agarose, bovine serum albumin (BSA), dithiothreitol, iodoacetamide, dansyl chloride, glycopeptidase-*N*-glycosidase (PNGase F), high molecular weight protein standards (205,000-29,000 daltons), aldolase and N-acetyl-L-tyrosine ethyl ester, were purchased from the Sigma Chemical Co., St.Louis, MO., USA. Wheat germ agglutinin was obtained from E.Y. Labs Inc., San Mateo, California, USA. Chymotrypsin A4 (from bovine pancreas) was obtained from Boehringer Mannheim, Gmbh, Germany. Sodium dodecyl sulphate, nitrocellulose sheets, acrylamide, bis-acrylamide, SDS-PAGE standards (14,500-94,000 daltons), TEMED, and Coomassie brilliant blue were purchased from Bio-Rad Laboratories, Richmond California, USA. ^{125}Iodine was obtained from Edmonton Radiopharmaceuticals, Edmonton, Alberta, or from the Amersham Corporation, Arlington Heights, Illinois. Sephadex G-25, Sephadex G-100, activated CH-Sepharose, ribonuclease A, and ovalbumin were purchased from Pharmacia Biotechnology, Dorval, Quebec. Kodak X-omat AR X-ray film was purchased from Eastman Kodak Co., Rochester, N.Y., USA. The round-bottomed microtiter plates for the hemagglutination inhibition assays (HAI)

were obtained from Flow Laboratories, McLean, Virginia, USA. The goose blood used for the HAI assays was purchased from Gibmar Laboratories, Ardrossen, Alberta. The plastic "Sealobags" used to incubate the nitrocellulose sheets with blocking buffer and ^{125}I-PT in the western blot procedure were purchased from Philips Electronics Ltd., Scarborough, Ontario.

2.2 Ceruloplasmin Isolation And Characterization.

Ceruloplasmin isolation and confirmation of purity was performed by the Connaught Research Institute for Biotechnology Research. Briefly, ceruloplasmin was purified [as suggested by Morell *et al.* (1969)] from 160 g of Cohn Fraction IV-1 of citric acid-trisodium citrate-dextrose-treated human plasma. The fraction was suspended in one litre of buffer containing 150 mM NaCl and 10 mM sodium acetate, pH 5.5. After heat-treating at 65°C for 18 h, the fraction was stored at 4°C overnight, and then was centrifuged at 11,000 *x g* for 30 minutes using a Sorvall R-2B centrifuge. Ceruloplasmin in the supernatant solution was further purified using DEAE-Sepharose CL-6B according to the method described by Morell *et al.* (1969). The ceruloplasmin was then concentrated by 60% $(NH_4)_2SO_4$ precipitation. The precipitate was dissolved in 0.1 M $NaHCO_3$ containing 0.5 M NaCl and dialyzed five times against the same buffer at 4°C. The ceruloplasmin solution was adjusted to a concentration of 5 mg/ml using the Bio-

Rad protein assay to determine the amount of protein, and stored at -20°C. The purity of the ceruloplasmin was confirmed by two dimensional double immunodiffusion (Ouchterlony technique; Kimball, J.W. 1986), using a polyclonal antiserum to ceruloplasmin, and polyclonal serum to other serum proteins.

2.3 Amino Acid Sequence Analysis Of The 72K (67K) Ceruloplasmin Fragment.

The determination of the amino acid sequence of the 72K (67K) ceruloplasmin fragment was done by the Mike Carpenter of the Department of Biochemistry, University of Alberta (Edmonton, Alberta), using an automated Protein Sequenator (Edman, P., 1950; Edman, P., and Begg, G., 1967; Hewick, R.M., *et al.*, 1981). Briefly, after initial coupling to prepare the phenylthiocarbamylated peptide, subsequent cleavage of the derivatized peptide resulted in the release of the 2-anilino-5-thiozolinone derivative of the terminal amino acid from the truncated peptide. The 2-anilino-5-thiozolinone derivative was then converted to a 3-phenyl-2-thiohydantoin (PTH) derivative (absorption maximum at 269 nm) and identified by reverse phase high performance liquid chromatography (HPLC). This series of reactions was repeated with the truncated peptide in order to identify the next amino acid in the sequence.

2.4 Preparation Of Sepharose-immobilized Pertussis Toxin.

Pertussis toxin was covalently attached to activated CH-Sepharose 4B in a ratio of 100 µg of toxin to 0.033 g of activated CH-Sepharose 4B (according to the manufacturer's instructions). CH-Sepharose couples to proteins via a N-hydroxysuccinamide condensation reaction, to form a peptide bond between free amino groups on the protein and N-hydroxysuccinamide groups esterified to the carboxyl end of a 6 carbon spacer arm on the Sepharose matrix. The gel was swollen in ice cold 1 mM HCl on a sintered glass funnel. The moist, HCl-washed CH-Sepharose was then placed in a 5 ml screw-capped vial that had been siliconized with a 5% solution of dimethyldichlorosilane in chloroform. Next, a solution of pertussis toxin dissolved in 0.1 M sodium bicarbonate (pH 8.0) (sonicated just prior to use for 30 seconds in a Branson model B-220 ultrasonic water bath to break up aggregates) was added to the vial. The mixture was incubated for 2 h at room temperature on a slowly-turning, end-over-end rotator. Following incubation, the toxin-agarose suspension was transferred to a sintered glass filter and washed with 50 ml of 50 mM Tris-HCl (pH 8.0) containing 0.5 M NaCl. The filtrate solution was collected and adjusted to pH 7 with HCl. The amount of PT in the neutralized filtrate was determined by measuring the optical density at a wavelength of 280 nm. This concentration estimate was based on a molar extinction

coefficient for pertussis toxin of approximately 2.0 (Arai, H., and Munoz, J.J. 1981). The filtrate was also analyzed for hemagglutinating activity using chymotrypsin-treated goose erythrocytes as described previously (sections 2.5 and 2.6; Armstrong, G.D. and Peppler, M.S., 1987). After calculating the amount of PT in the filtrate, it was possible to determine indirectly the amount of PT that had become covalently attached to the agarose. The washed PT-agarose was then incubated in 3 ml of 1 M ethanolamine (pH 9) for 1 hour to inactivate any remaining N-hydroxysuccinamide groups on the CH-Sepharose. After removing the ethanolamine solution, the PT-agarose was briefly incubated in 3 ml of the Tris-HCl wash buffer and approximately 0.5 ml of the Pertussis toxin-sepharose suspension was transferred to a glass wool-plugged Pasteur pipet. The affinity column was washed alternately with 20 volumes of 0.1 M sodium acetate buffer (pH 4) and Tris-HCl wash buffer. Finally, the column was washed with 20 volumes of 0.1 M phosphate-buffered saline (pH 7.2) physiological saline (0.15 M NaCl) containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS). The columns were stored at 4°C in the presence of PBS containing 0.1% sodium azide.

2.5 Chymotrypsin Treatment Of Goose Erythrocytes.

The procedure was described previously (Armstrong, G.D., and Peppler, M.S. 1987) and was designed to increase the sensitivity of the PT-mediated agglutination of goose

erythrocytes. Briefly; two hundred µl of fresh goose blood (Gibmar Laboratories) diluted 1:1 in Alsever's buffer solution (consisting of 2.05 g of glucose, 0.8 g of sodium citrate, 0.42 g of sodium chloride, and 0.055 g of citric acid, per 100 ml of water) was suspended in 10 ml of PBS. The erythrocytes were then centrifuged at 200 x *g* for 5 min, and the supernatant solution was carefully decanted. The washed erythrocytes were then suspended in 0.9 ml of PBS. Chymotrypsin (0.1 ml of a 10 mg/ml solution) was then added to give a final concentration of 1 mg/ml, and the suspension was incubated at 37°C for 30 minutes. Next, the chymotrypsin-treated erythrocytes were centrifuged (as described above), and the cells were washed four times with 10 ml of PBS to remove the remaining chymotrypsin. The treated erythrocytes were suspended in 10 ml of PBS just prior to use.

2.6 Hemagglutination (HA) Assay To Quantitate Binding Activity Of Native And Iodinated Pertussis Toxin Or Wheat Germ Agglutinin.

The procedure has been described previously (Armstrong, G.D., and Peppler, M.S. 1987). Unlabeled PT or WGA was suspended to a concentration of 1 µg/ml in PBS. ^{125}I-PT or ^{125}I-WGA were diluted 10 times in PBS. Both solutions were sonicated for 30 seconds prior to use in a Branson model B-220 ultrasonic water bath. Subsequently, serial two-fold dilutions of the toxin or lectin

preparations were prepared in PBS, and 50 µl of each dilution was added to the appropriate wells in 96-well round-bottomed microtitre plates. Chymotrypsin-treated goose erythrocytes were suspended at a concentration of approximately 2 x 10^7 cells/ml, and 50 µl was added to each of the wells. Following incubation at room temperature for 2 h, the last well demonstrating HA (cells spread evenly over bottom of well), was taken as the endpoint titre, and the concentration of the ^{125}I-PT or ^{125}I-WGA was determined by comparing its end-point titre to that of the unlabeled PT or WGA of known concentration.

2.7 Reduction And Alkylation Of Sulfhydryl Groups In Ceruloplasmin.

Ultrapure urea (2.4 g; final concentration = 8 M) crystals were added to native ceruloplasmin (28 mg) in 5 ml of 0.4 M sodium phosphate (pH 8.3), and the pH was adjusted to 8.5 with 1 M NaOH. The resulting solution was then incubated at room temperature with stirring for 1 h under nitrogen. Next, dithiothreitol (80 mg) was added and the solution was further incubated with stirring under nitrogen for an additional 4 h at room temperature. Iodoacetamide (0.2 g) was then slowly added while maintaining the pH as close as possible to 8 using 1.0 M NaOH. The reduced and alkylated (R/A) protein was dialized for 20 h against H_2O obtained from a Milli-Q water purification system (changing the water several times) to remove all of the salt. The

salt-free protein solution was poured into a 100 ml round bottomed flask and the flask was slowly rotated in a dry-ice/methanol bath until the solution was completely frozen. The reduced and alkylated (R/A) ceruloplasmin was then lyophilized for 14 h using a New Brunswick freeze drying apparatus.

2.8 Analysis Of Ceruloplasmin By Affinity Chromatography And Discontinuous Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Prior to its use, the fetuin agarose was sedimented by centrifugation at 100 x *g* for 2 minutes and after discarding the supernatant solution, the resulting pellet was washed once with ten volumes of 4 M magnesium chloride. The mixture was again centrifuged at 100 x *g*, and the magnesium chloride supernatant solution was decanted off. The pellet of fetuin-agarose was subsequently washed four times with PBS, with the pellet being collected after each wash by centrifugation (as described above). A small column of fetuin-agarose was prepared by placing approximately 0.5 ml of the suspension into a glass wool-plugged Pasteur pipet as described earlier (section 2.4). Fetuin agarose was used as a control for non-specific binding in the affinity chromatography experiments. Native and R/A ceruloplasmin (50 µl; 5.6 mg/ml) samples were slowly applied to the agarose-immobilized Pertussis toxin

(section 2.4) or fetuin-agarose. Once the sample had drained into the agarose, the flow of buffer was stopped for 15 min to allow the ceruloplasmin fragments to bind. The columns were then washed with 1.0 ml of PBS, during which time twenty 50 µl fractions were collected from each column. Next, bound protein was eluted using 0.5 ml of a solution of 0.15 M NaCl and 50 mM diethanolamine (pH 11.5). The diethanolamine was chosen because of its ability to disrupt PT-glycoprotein interactions (Chong and Klein, 1989). After collecting ten 50 µl aliquots, fifty µl of SDS-PAGE sample buffer (no mercaptoethanol) was added to every fourth PBS-wash fraction and to every second diethanolamine fraction. Five µl of 2-mercaptoethanol was added to each sample and the samples were heated in a boiling water bath for 15 minutes Twenty µl of fractions 1-20 (PBS wash fractions) and 50 µl of fraction 21-30 (diethanolamine eluate fractions) were then analyzed by discontinuous SDS-PAGE using a 12.5% separating gel and a 5.0% stacking gel as described previously (Laemmli, U.K., 1970). The gels were fixed overnight in a solution of 40% ethanol, 5% acetic acid. Proteins were visualized using the silver stain procedure described by Tsai, C.M., and Frasch, C.E., (1982).

2.9 Preparation Of Iodo-Gen Tubes.

Twenty µl of Iodo-Gen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril) dissolved in chloroform at a

concentration of 2.0 mg/ml, was added to the bottom of chromic acid-washed 12 x 75 mm glass culture tubes. The tubes were slowly agitated using a Vortex mixer, while applying a gentle stream of nitrogen gas to evaporate the chloroform. This procedure caused the deposition of concentric rings of insoluble Iodo-Gen in the bottom of the tubes.

2.10 Iodination Of Pertussis Toxin In The Presence Of Fetuin Agarose.

Biologically active, ^{125}I-labeled Pertussis Toxin (PT), was prepared by the fetuin-agarose procedure described previously (Armstrong, G.D., and Peppler, M.S., 1987), with some modifications. After washing the fetuin-agarose as described in section 2.8, 50 µl of the pellet was placed in the bottom of an Eppendorf microfuge tube and 20 µl of PT (0.3 µg/µl) in sodium phosphate buffer; pH 8.3 was added. The preparation was gently mixed, then incubated for 30 minutes at room temperature on an end-over-end rotator to allow the PT to bind to the fetuin. Next, 3.7 MBq of sodium ^{125}iodine was carefully added to the gel slurry, and after gentle mixing, the entire preparation was transferred to the bottom of an Iodo-Gen tube (preparation described in section 2.9). After allowing the iodination reaction to proceed for 1 minute, 200 µl of 1 µg/µl cysteine was added to the mixture to quench the reaction. Next, 1 ml of PBS was added to the Iodo-Gen

tube, and the entire slurry was transferred to a Pasteur pipet plugged with a small amount of glass wool. Approximately 20 ml of 10 mM potassium iodide in PBS was then used to remove unincorporated ^{125}iodine from the fetuin-agarose-PT preparation. The remaining potassium iodide wash solution was gently pushed out of the Pasteur pipet with a small rubber bulb. Two 100 µl aliquots of 50 mM diethanolamine solution containing 0.15 M NaCl (pH 11.5) were added to elute the ^{125}I-PT from the fetuin-agarose. The ^{125}I-PT was collected in a 12 x 75 mm glass culture tube containing 200 µl of PBS, 100 µl of 1 mg/ml cysteine, and 7 µl of 1M HCl (to immediately neutralize pH of the solution). The percentage of counts incorporated into protein was then determined using the TCA precipitation procedure described in section 2.13.

2.11 Preparation Of Sephadex G-25 Gel Filtration Column.

When preparing ^{125}I-Wheat germ agglutinin, it was necessary to separate free ^{125}iodine from the iodinated protein using a Sephadex G-25 gel filtration column. Briefly, fifteen ml of PBS was added to 2 g of Sephadex G-25 and the mixture was incubated at room temperature for 15 minutes. The top of a 10 ml disposable glass pipet was then cut off and a small wad of glass wool was gently pushed into the tip of the pipet. A short piece of Tygon tubing was placed on the tip of the pipet and clamped shut.

The pipet was fixed to a ring stand and a glass funnel was attached to the top with a small piece of rubber tubing. The entire assembly was filled with PBS, care being taken not to trap any air bubbles. The clamp was then opened to allow some of the PBS to drain from the column When the level of PBS had reached the bottom of the funnel, the flow was stopped, and the swelled Sephadex slurry was poured into the funnel. After allowing the gel to settle to the bottom of the column (5 minutes), the clamp was reopened and the flow rate was adjusted to approximately 1 drop per second. After allowing the column to pack, the funnel was removed. If the column was not prepared for immediate use, the top was sealed with Parafilm and the column was stored at room temperature.

2.12 Iodination Of Wheat Germ Agglutinin By Iodo-Gen Procedure.

Wheat germ agglutinin (WGA) was iodinated by the Iodo-Gen procedure described previously (Armstrong, G.D., et al., 1982), with some modifications. Briefly, forty µl of WGA (1 µg/µl) in PBS and 160 µl of *N*-Acetylglucosamine (0.4 M) was placed in the bottom of a 12 x 75 mm glass culture tube for 10-15 minutes at room temperature. The iodination reaction was performed in the presence of *N*-acetylglucosamine (GlcNAc) to prevent damage to glycoprotein binding sites on the WGA. After transferring the solution to an Iodo-Gen tube, 11.1 MBq of sodium

^{125}iodine was added, and the mixture was allowed to react for 2 minutes. Next, the preparation was passed through a glass-wool-plugged Pasteur pipet into a clean 12 x 75 mm tube. To separate free ^{125}iodine from ^{125}I-WGA, a Sephadex G-25 column was used (preparation described in section 2.11). The buffer from the top of the G-25 column was first removed and 0.5 ml of PBS containing 1.0% BSA was added. The clamp on the Tygon tubing was subsequently opened to allow the BSA to drain into the gel bed. The iodinated mixture was then added to the column and allowed to drain into the gel. This fraction was collected in a 12 x 75 mm glass culture tube (tube 1). Next, 0.5 ml of PBS-1.0% BSA was added to the column after the radioactive sample and also allowed to drain into the gel while collecting the flow through (tube 2). After filling the column with PBS, I continued to collect 0.5 ml fractions. After collecting 16 fractions, 10 µl aliquots of each fraction were transferred to 12 x 75 glass culture tubes, and counted in an LKB Rackgamma Counter, to determine the location of ^{125}I-WGA in the void volume. Fractions containing ^{125}I-WGA were then pooled, and the concentration of ^{125}I-WGA that retained its binding activity was estimated using the hemagglutination assay (section 2.6). The ^{125}I-WGA preparation could be stored (in 0.5 ml aliquots) for several weeks at -90°C without losing binding activity.

2.13 Determination Of Percent Incorporation Of ^{125}I Into Protein.

Determination of the number of ^{125}Iodine counts incorporated into PT or WGA was accomplished by trichloroacetic acid (TCA) precipitation as described earlier (Armstrong, G.D., and Peppler, M.S., 1987). Briefly, 5 µl aliquots of the iodinated preparations were added to 1.0 ml of PBS-1% BSA (duplicate tubes were prepared). TCA (0.5 ml of a 10% solution) was added to one of the tubes [(+) TCA], and PBS (0.5 ml) was added to the other [(-) TCA]. Both tubes were vigorously mixed using a Vortex mixer, and placed on ice for at least ten minutes. Both tubes were then centrifuged at 700 x *g* for 10 minutes, to sediment the protein precipitated by the addition of TCA. The amount of radioactivity incorporated into protein was then computed from the counts remaining in the supernatant solutions of these tubes. Radioactivity was recorded in an LKB Rackgamma model 1270 gamma counter. The number of counts covalently bound to protein was consistently greater than 60% in the ^{125}I-PT preparations, and 80% in the ^{125}I-WGA preparations.

2.14 ^{125}I-Pertussis Toxin And ^{125}I-Wheat Germ Agglutinin Fetuin-coated Polystyrene Tube Binding Assays.

The polystyrene tube binding assay was performed as described previously (Sekura, R., *et al.*, 1985) but with

some modifications. One hundred µl of fetuin solution (50 µg/ml in sodium phosphate buffer, pH 6.8) was carefully placed into the bottom of each 12 x 75 mm polystyrene tube (Fisher Scientific). The tubes were incubated overnight at room temperature in a water saturated environment to prevent evaporation. The fetuin coating solution was then removed from the tubes by aspiration and the tubes were washed with three successive 0.5 ml portions of PBS (10 minutes incubation between each wash cycle). Five Hundred µl of PBS containing 5% BSA was then placed in the bottom of each tube to saturate the unoccupied protein binding sites (4 h incubation at room temperature required). The BSA blocking solution was removed by aspiration, and the tubes were again washed with three 0.5 ml portions of PBS. Next, 80 µl of PBS containing 1% BSA was carefully placed in the bottom of each tube and to this was added 10 µl of varying concentrations of inhibiting glycoprotein or glycopeptide dissolved in PBS-0.1% BSA. ^{125}I-labeled PT, was prepared by the fetuin-agarose procedure described previously (section 2.10; Armstrong, G.D., and Peppler, M.S., 1987). Wheat germ agglutinin (WGA) was iodinated by the Iodo-Gen procedure described previously (section 2.12; Armstrong, G.D., *et al.*, 1982). Sonication of the ^{125}I-PT in a Branson model B-220 ultrasonic water bath just prior to use was necessary to break up aggregates. In the PT-ceruloplasmin tube inhibition assays, 10 µl of the ^{125}I-PT preparation was added to each tube. The final

concentration of labeled PT or WGA was approximately 1 x 10^{-9} M in all assays. The solutions were incubated overnight at 4°C in a water saturated environment. The labelled PT and WGA solutions were then aspirated from the tubes, and unbound radioactivity was removed by four successive washes with ice-cold 1% BSA in PBS (1.0 ml per wash) with 4-5 seconds of "vortexing" between each wash. The amount of bound radioactivity in each tube was determined in an LKB Rack Gamma model 1270 gamma counter. Background binding of ^{125}I was determined in BSA-saturated polystyrene tubes that had not been pre-coated with fetuin (100 µl of 5% BSA in PBS was used). Statistical analysis of the data was performed as suggested previously (Skoog, D.A., and West, D.M. 1969).

2.15 Limited Enzymatic Cleavage Of Ceruloplasmin Using Trypsin And Chymotrypsin.

Trypsin, (50 µl; 1.0 mg/ml in 100 mM Tris-HCl buffer pH 8.3) was incubated with native ceruloplasmin (4.5 ml; 5.6 mg/ml) for 18 h at 37°C. A portion (0.5 ml) of the trypsin-treated ceruloplasmin was incubated for an additional 18 h at 37°C (trypsin control) and a second 0.5 ml portion was incubated with Chymotrypsin (5.0 µl; 1.0 mg/ml). The native and protease-treated ceruloplasmin samples were analyzed by discontinuous SDS-PAGE using a 12.5% separating gel and a 5.0% stacking gel as described previously (Laemmli, U.K., 1970). The gels were fixed

overnight in 40% ethanol, 5% acetic acid, and stained with the Coomassie Brilliant Blue R-250 stain consisting of 2.5 g of dye (obtained from Bio-Rad laboratories), 0.5 L Methanol, 0.1 L Glacial Acetic Acid, 0.4 L distilled water, thoroughly stirred and filtered (Whatmann #4 filter) prior to use. The gels were destained by incubating them in a solution containing 10% Methanol and 10% Acetic Acid.

2.16 Analysis Of Ceruloplasmin Peptides By The "Western" Blot Procedure.

Enzymatically treated or untreated ceruloplasmin peptides were analyzed by discontinuous SDS-PAGE (in duplicate) using a 10% or 12.5% separating gel. One of the duplicate SDS-PAGE gels was fixed, and stained with Coomassie blue, and the other was used in the western blot procedure. In preparation for western blotting, several pieces of Whatmann #1 filter paper and a sheet of Bio-Rad nitrocellulose (cut approximately to the size of gel) were placed into transfer buffer (11.50 g of Na_2HPO_4 and 4.58 g of NaH_2PO_4 in water obtained from a Milli-Q water purification system) for at least 15 minutes prior to use. Immediately after electrophoresis, the unfixed, unstained gel was placed on three layers of pre-wetted Whatmann #1 filter paper. After any air bubbles between the gel and filter paper were carefully removed, the wet nitrocellulose sheet was placed on top of the gel. Again, all visible air bubbles were removed. Three additional layers of pre-

wetted Whatmann #1 filter paper were placed on top of the nitrocellulose sheet. This entire process was performed with gloved hands (to avoid contamination of the blot) in a large tray containing transfer buffer, to prevent the formation of any air bubbles which would interfere with the complete transfer of peptides from the gels to the nitrocellulose sheets. The entire "sandwich" was then placed in the Bio-Rad Trans-Blot holding apparatus between two "Brillo" pads, and the completed assembly was placed in the Bio-Rad Trans-Blot cell (filled with transfer buffer). The peptides were electrophoretically transferred to nitrocellulose sheets (Burnette, W.N., 1981) by applying a current of 27 volts overnight. The temperature was moderated using a circulating cooling system, operating at 10°C. Following peptide transfer, the blots were stained with amido black to ensure complete peptide transfer. In addition, the blotted gels were routinely stained with the Coomassie blue stain, to confirm that the majority of peptides had been transferred to the nitrocellulose. The blots were placed in a Philips "Sealobags" and 30 ml of BSA-blocking buffer (5% bovine serum albumin in 10 mM Tris-HCl, 0.15 M NaCl, 0.1% Na-azide, 5% BSA, pH 7.5), was added. The bags were heat-sealed, and incubated on a slowly turning end-over-end rotator overnight at 4°C to saturate unoccupied protein binding sites on the nitrocellulose sheets. The blocking buffer was removed, and the nitrocellulose blots were incubated with 30 ml of

fresh blocking buffer containing ^{125}I-labeled PT (iodinated as described previously in section 2.10; Armstrong, G.D., and Peppler, M.S., 1987) overnight on the end-over-end rotator at 4°C. Prior to its addition to the blots, the ^{125}I-PT was sonicated and filtered through a 0.22 µm filter into fresh blocking buffer. This was necessary to break up aggregates which would otherwise contribute to excessive background binding. After overnight incubation, the radioactive solution was drained from the "Sealobag" and the blot was washed nine times using 200-250 ml of 0.85% NaCl in H_2O (10 minutes incubation between wash cycles), air dried, and exposed to Kodak X-OMAT AR film for 4 h at -90°C using DuPont Cronex Lightning Plus intensifying screens (Burnette, W.N., 1981). To demonstrate the binding specificity of iodinated PT on western blots, duplicate blots of native ceruloplasmin peptides were prepared. One blot was incubated with 0.4 µg of biologically active ^{125}I-PT alone (~100000 cpm), and another blot was incubated with ^{125}I-PT in the presence of 2000 fold excess (800 µg) unlabeled PT.

2.17 Removal Of *N*-linked Oligosaccharide Units From R/A Ceruloplasmin With Glycopeptidase-*N*-glycosidase (PNGase F).

R/A ceruloplasmin (70 µg) was added to 0.11 ml of 200 mUnit/ml (22 mUnit) PNGase F in 20 mM sodium phosphate buffer (pH 7.2) containing 50 mM EDTA, 0.1% SDS and 1% 2-

mercaptoethanol. The solution was incubated without shaking overnight at 37°C to remove *N*-linked oligosaccharides from the ceruloplasmin (Plummer, T.H., *et al.*, 1984). R/A ceruloplasmin and the PNGase F-treated ceruloplasmin were analyzed by SDS-PAGE and analyzed by the western blot procedure as described in section 2.16.

2.18 Dansyl Chloride Labeling Of Ceruloplasmin And Elution Of Ceruloplasmin Bands From Non-fixed (NF), Non-stained (NS) Polyacrylamide Gels.

Dansyl chloride (10 µl of a 20% solution in acetone) was added with vigorous shaking to native ceruloplasmin (18 µl; 5.6 µg/µl) in 3% SDS, 0.10 M Tris-acetate buffer (pH 8.2) and incubated for 1 week at room temperature. The dansylated ceruloplasmin samples (DNS-ceruloplasmin) were mixed with native ceruloplasmin in a ratio of 1:5 and separated by SDS-PAGE using a 10% separating gel. Following electrophoresis, DNS-ceruloplasmin bands were visualized in the gels using an ultraviolet light box (340 nm). The fluorescent bands were cut out of the gel, and rolled with a gloved finger on a glass plate to break the gel slices into small pieces. The gel pieces were then put into a 13 x 100 mm test tube, and 3.0 ml of elution buffer was added (50 mM Tris, 0.1% SDS, 0.1 mM EDTA, 5 mM DTT, 0.20 M NaCl, pH 7.9; Hager, D.A., and Burgess, R.R. 1980). Following overnight incubation at room temperature on an end-over-end rotator, the gel slurry was centrifuged at 200

x *g* for ten minutes and the supernatant solution containing the peptides was collected. To increase recovery, an additional 3 ml of elution buffer was added to the pieces of acrylamide, and the samples were further incubated on an end-over-end rotator for 2 h, centrifuged and the supernatant solutions again collected. The supernatant solutions were combined and filtered using a Millipore 0.22 µm filter to remove any contaminating gel or bacteria. The filtered samples were then concentrated using an 8010 Amicon ultrafiltration system fitted with a 25 mm YM 10 filter (molecular weight cutoff <10,000). Next, the samples were dialyzed against H_2O (obtained from a Milli-Q water purification system), and the protein concentration was determined using the Lowry assay (Lowry, O.H., *et al.*, 1951). The ceruloplasmin peptides were analyzed by comparing their banding pattern with that of native ceruloplasmin using a Bio-Rad SDS-PAGE MiniGel apparatus, with a 12.5% separating gel (the gel was 0.5 mm thick). The gels were stained with Coomassie Brilliant Blue R-250. These peptides were then used in the fetuin-coated polystyrene tube inhibition assay described earlier.

2.19 Analysis Of Ceruloplasmin And Ceruloplasmin Fragments By Gel Filtration.

To examine the molecular weights of the R/A ceruloplasmin and the individual ceruloplasmin fragments (67K/72K and 50K fragments) in solution, a Sephadex G-100

column was prepared. Briefly, 17 g of Sephadex G-100 was incubated with 0.5 l of PBS for 1 h, and the slurry was poured into a Pharmacia C column (internal diameter of 26 mm, a length of 70 cm, and a volume of 361 ml), with an attached overflow adapter (1 l volume). After allowing the gel to settle overnight, the bed height was adjusted to approximately 67 cm, by removing excess gel slurry. The column was calibrated by determining the elution volume of protein standards of known molecular weight. The column flow rate during elution of proteins was approximately 0.3 ml/min. Fractions were collected using an LKB 2070 ultrorac II apparatus, equipped with a model 2132 microperpex peristaltic pump, and a model 2238 uvicord S II detector. A model 2210 (2 channel) strip chart recorder was used to record the results.

3.0 RESULTS

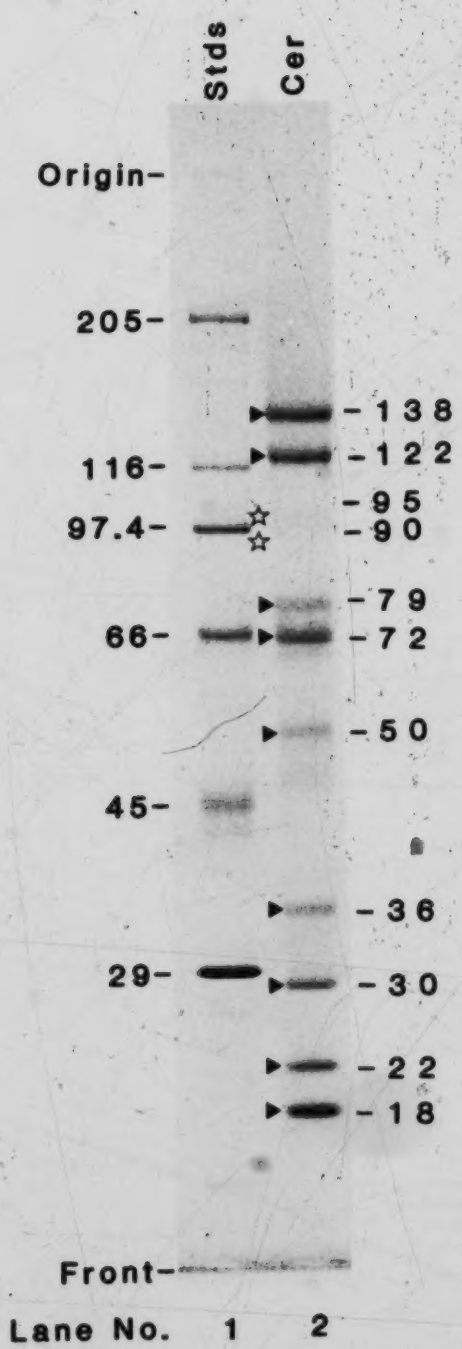
3.1 SDS-PAGE Analysis Of Ceruloplasmin.

In this experiment, SDS-PAGE was carried out using a 7.5% to 15% acrylamide gradient separating gel. In addition, Sigma high molecular weight standards (205,000-29,000 daltons) were used to better resolve ceruloplasmin fragments and achieve a more accurate estimate of their molecular weight. The major ceruloplasmin bands had molecular weights of 138K, 122K, 79K, 72K, 50K, 36K, 30K, 22K and 18K (figure 4). There were also minor ceruloplasmin fragments at 95K and 90K.

3.2 Affinity Chromatography Analysis Of Ceruloplasmin Using Pertussis Toxin Affinity Columns.

The previous SDS-PAGE analysis of ceruloplasmin demonstrated a variety of ceruloplasmin fragments, and it was hoped that the PT affinity isolation procedure would enable me to identify those fragments with PT-binding activity. After washing unbound ceruloplasmin fragments from the PT-affinity column, bound peptides were eluted with diethanolamine. Analysis of the PBS (wash) and diethanolamine (eluted) fractions by discontinuous SDS-PAGE using a 12.5% separating gel (and subsequent silver staining) demonstrated a variety of ceruloplasmin peptides in the diethanolamine fractions (figure 5). Native and R/A

Figure 4. SDS-PAGE Analysis Of Ceruloplasmin Peptides. Native ceruloplasmin peptides were separated by discontinuous SDS-PAGE using a 5% stacking gel and a linear gradient separating gel (7.5-15%). The gel was then stained with Coomassie Brilliant Blue R-250. The positions to which the molecular weight standards migrated are marked on the left side of the panel. Major (▶) and minor (☆) ceruloplasmin bands are marked to the left of their positions.

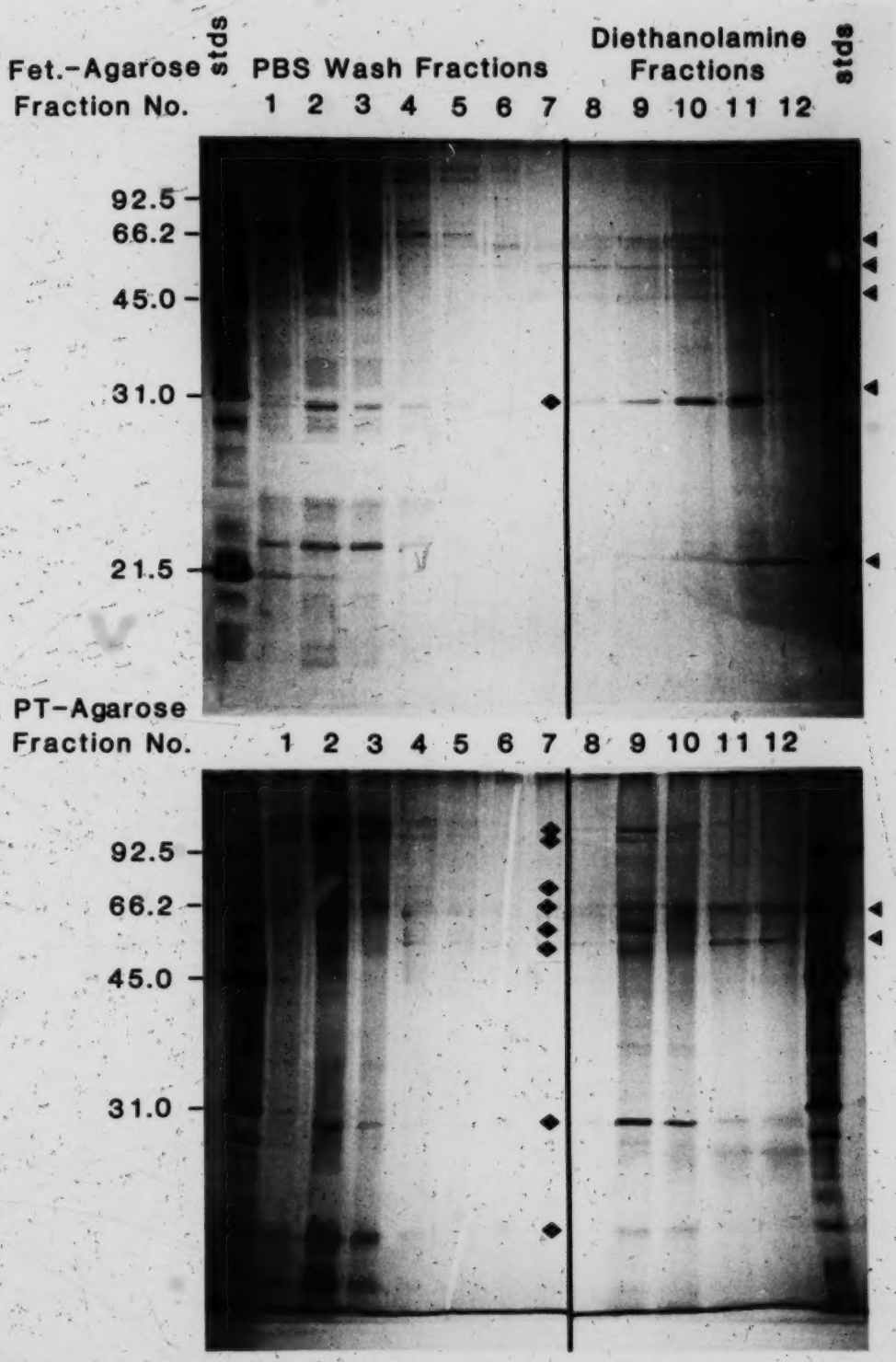

Stds
Cer
Origin-
205-
116-
97.4-
66-
45-
29-
Front-
Lane No. 1 2
138
122
95
90
79
72
50
36
30
22
18

ceruloplasmin fragments with apparent molecular weights of 138K, 122K, 90K, 79K, 72K, 50K, 30K and 22K were found. To identify peptides non-specifically interacting with the agarose, a native ceruloplasmin sample was applied to a fetuin-agarose column. Subsequent analysis by discontinuous SDS-PAGE revealed that the 30K peptide appeared in the fractions eluted with diethanolamine.

3.3 Labeling Of Native Ceruloplasmin With Dansyl Chloride.

Native ceruloplasmin was labelled with Dansyl chloride (DNS-Cl) so that the individual ceruloplasmin fragments could be visualized (using an ultraviolet light source) on non-fixed, non-stained polyacrylamide gels. This was necessary for obtaining purified fragments by the preparative SDS-PAGE procedure (section 2.18). Following separation of the DNS-Cl-labelled ceruloplasmin by SDS-PAGE, the fluorescent areas of the gel containing the individual ceruloplasmin fragments (molecular weights of 138K, 122K, 79K, 72K, and 50K; figure 6), were cut from the gel. The peptides were subsequently eluted from the gels using a SDS-Tris buffer. After dialyzing the sample against H_2O, and determining the concentration of protein by the Lowry assay, the isolated ceruloplasmin fragments were separated by SDS-PAGE to confirm purity of the sample. The peptides were subsequently used in the western blot analysis (section 3.4 and figure 12) and in the fetuin-

Figure 5. Affinity Isolation Analysis Of Native Ceruloplasmin Peptides. Affinity chromatography was used to identify fetuin-agarose-bound (top panel) and PT-agarose-bound (lower panel) ceruloplasmin peptides. Native ceruloplasmin peptides were passed through glass wool-plugged Pasteur pipets containing approximately 1 mL of fetuin- or PT-agarose. The fractions were then analyzed by discontinuous SDS-polyacrylamide gel electrophoresis (+2-mercaptoethanol), using a 12.5% separating gel. Following electrophoresis, the gels were fixed overnight in 40% ethanol-10% acetic acid, stained by the silver stain procedure and photographed. Lanes 1-7 contain peptides that did not bind to fetuin- or PT-agarose. Lanes 8-12 contain peptides eluted from the fetuin- or PT-agarose using 50 mM diethanolamine, 0.15 M NaCl (pH 11.5). Bands marked by filled triangles on the right represent artifacts that may be due to spill-over of the molecular weight standards. Bands marked by filled diamonds to the left of the dividing line represent ceruloplasmin peptides. The positions to which the molecular weight standards migrated are marked to the left side of each panel.

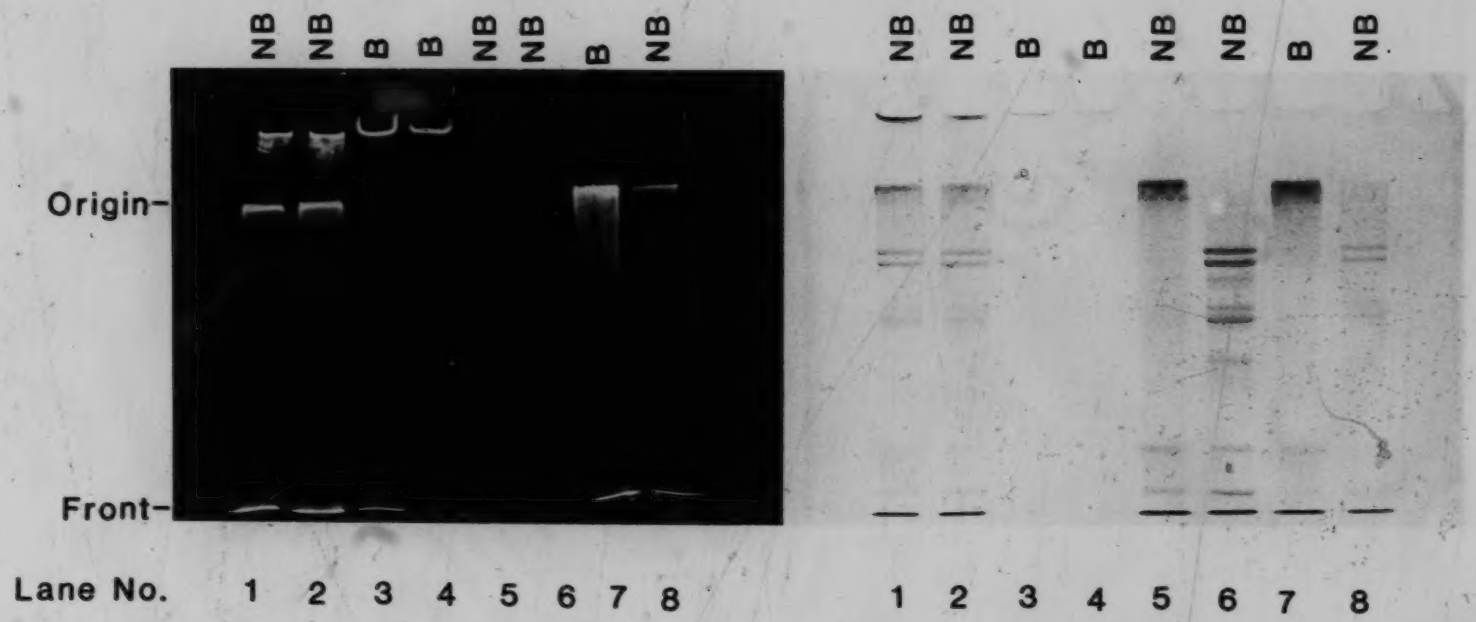

Fet.-Agarose
stds
PBS Wash Fractions
Diethanolamine Fractions
stds
Fraction No.
1 2 3 4 5 6 7 8 9 10 11 12
92.5
66.2
45.0
31.0
21.5
PT-Agarose
Fraction No.
1 2 3 4 5 6 7 8 9 10 11 12
92.5
66.2
45.0
31.0

coated polystyrene tube inhibition assay (section 3.5.2 and figure 14).

3.4 Western Blot Analysis Of Ceruloplasmin.

The western blot containing the native and R/A ceruloplasmin peptides stained with amido black confirmed that complete peptide transfer had occurred to the nitrocellulose sheets (figure 7, panel A). The banding patterns of the R/A ceruloplasmin samples were identical regardless of whether or not 2-mercaptoethanol was present in the Laemmli sample buffer (figure 7, lanes 2 and 5). Slightly different banding patterns were seen with the native ceruloplasmin samples in the presence and absence of 2-mercaptoethanol: a high molecular weight aggregate was seen at the top of the separating gel in the absence of 2-mercaptoethanol. After the blot containing native and R/A ceruloplasmin peptides was probed with ^{125}I-PT, several bands with PT-binding activity were seen. In the presence of 2-mercaptoethanol, the ^{125}I-PT bound to native and R/A ceruloplasmin fragments with molecular weights of 138K, 122K, 95K, 72K and to a lesser extent, to bands at 90K, 79K, and 50K (figure 7, panel B, lanes 2, 3, 5, and 6). The ^{125}I-PT also bound to the high molecular weight aggregate that appeared at the top of the separating gel when native ceruloplasmin was not treated with 2-mercaptoethanol prior to SDS-PAGE (figure 7, panel B, lane 6). In addition, the ^{125}I-PT apparently bound to the native

Figure 6. SDS-PAGE Analysis Of Dansyl Chloride (DNS-Cl)-treated Ceruloplasmin Peptides. Samples of native ceruloplasmin were dialyzed overnight in 0.1 M NaCl to remove ammonium salts, then further dialyzed against water 3x to remove the sodium chloride. Freshly prepared dansyl chloride (in acetone) was added to each tube. Lanes 1, and 2 represent ceruloplasmin samples incubated for one week with 2.0% DNS-Cl at room temperature (prior to SDS-PAGE analysis). Lanes 3 and 4 represent ceruloplasmin samples incubated with 2.0% DNS-Cl in a boiling water bath for 10 minutes. Lanes 5 and 6 represent ceruloplasmin samples incubated at room temperature (for one week) with all materials except DNS-Cl. Lane 7 represents a ceruloplasmin sample containing 0.1% DNS-Cl that was incubated in a boiling waterbath, whereas lane 8 represents a ceruloplasmin sample incubated at room temperature for one week with 0.1% DNS-Cl. In addition, the resulting samples were either boiled (lanes 1, 3, 5, 7, 8) or not boiled (lanes 2, 4, 6) prior to SDS-PAGE analysis (Bio Rad MiniGel) using a 10% separating gel. Panel A represents a fluorescent image of the unfixed, unstained gel. Panel B represents the gel stained with Coomassie Brilliant Blue. The "B" on panels A and B indicates the samples that were incubated in a boiling water bath with the DNS-Cl (either 2% or 0.1%), whereas the "NB" indicates the samples that were incubated with the DNS-Cl at room temperature for one week.



A. Fluorescence

B. Coomassie stained

and R/A ceruloplasmin samples that had been prepared for SDS-PAge analysis in the absence of 2-mercaptoethanol (figure 7, panel B, lanes 5 and 6).

The ^{125}I-PT and native PT demonstrated the same specificity for PT receptors on western blots in that the native PT was able to compete with the ^{125}I-PT for ceruloplasmin binding sites (figure 8). Duplicate ceruloplasmin blots were prepared and incubated with identical amounts of (0.4 µg) ^{125}I-PT, however, one blot was incubated with an excess (800 µg) of native PT. The blot incubated with the unlabeled PT (figure 8, lane 1) demonstrated a very low amount of binding by the ^{125}I-PT. The blot incubated with only the ^{125}I-PT, demonstrated a much higher degree of ^{125}I-PT-binding (figure 8, lane 2).

After the native ceruloplasmin samples were treated with proteolytic enzymes (trypsin and chymotrypsin), SDS-PAGE analysis revealed that most of the high molecular weight ceruloplasmin fragments were cleaved to lower molecular weight fragments (figure 9, panel A, lanes 3 and 4). Furthermore, the blot of these peptides demonstrated reduced PT-binding activity with the trypsin-treated ceruloplasmin. Ceruloplasmin bands with apparent molecular weights of 72K, 39K, 30K, 28K and 26K and a weakly interacting band at 122K were seen on autoradiograms of nitrocellulose blots (figure 9, panel B, lane 2). Amino terminal sequence analysis of the band at 72K demonstrated that this peptide was probably equivalent to the 67K N-

Figure 7. Analysis Of R/A Ceruloplasmin (R/A Cer) And Native Ceruloplasmin (Cer) By Discontinuous SDS-PAGE And Western Blotting Procedures. Ceruloplasmin peptides (native and R/A) were analyzed using a 12.5% separating gel and 5% stacking gel in the presence (+2-ME) and absence (-2-ME) of 2-mercaptoethanol (lane 1 contains the molecular weight standards). Panel A. Photograph of an amido black-stained gel of the ceruloplasmin peptides. Panel B. Autoradiograph of ^{125}I-PT probed blot: ceruloplasmin peptides were transferred electrophoretically to nitrocellulose sheets. After blocking the blots with BSA, the blot was incubated with ^{125}I-labeled Pertussis toxin overnight at 4°C. The blot was washed with 0.85% NaCl, air dried, and exposed to Kodak X-OMAT AR film for 4 h at -70°C using Cronex Lightning Plus intensifying screens. The ceruloplasmin fragments with high (filled triangles ▶) and low (filled circles ●) intensity PT binding interactions are marked to the right of their positions on panel A.

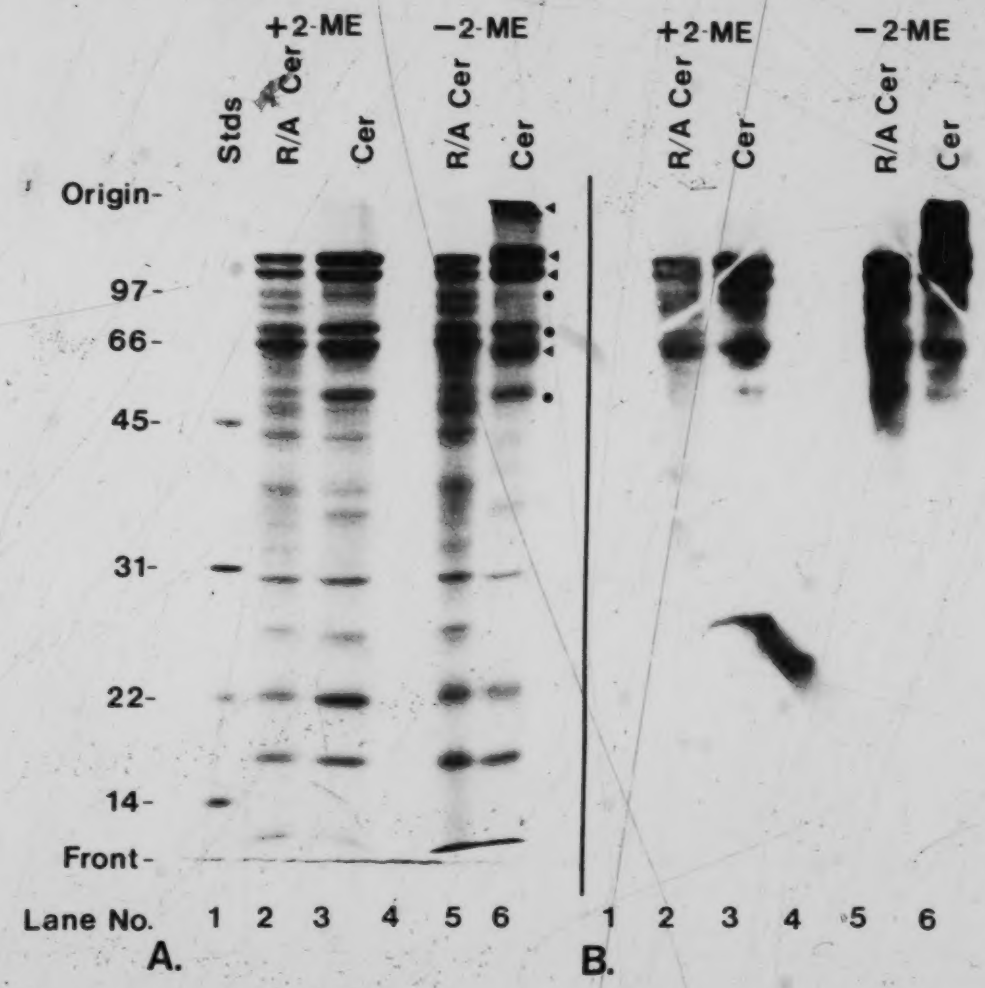
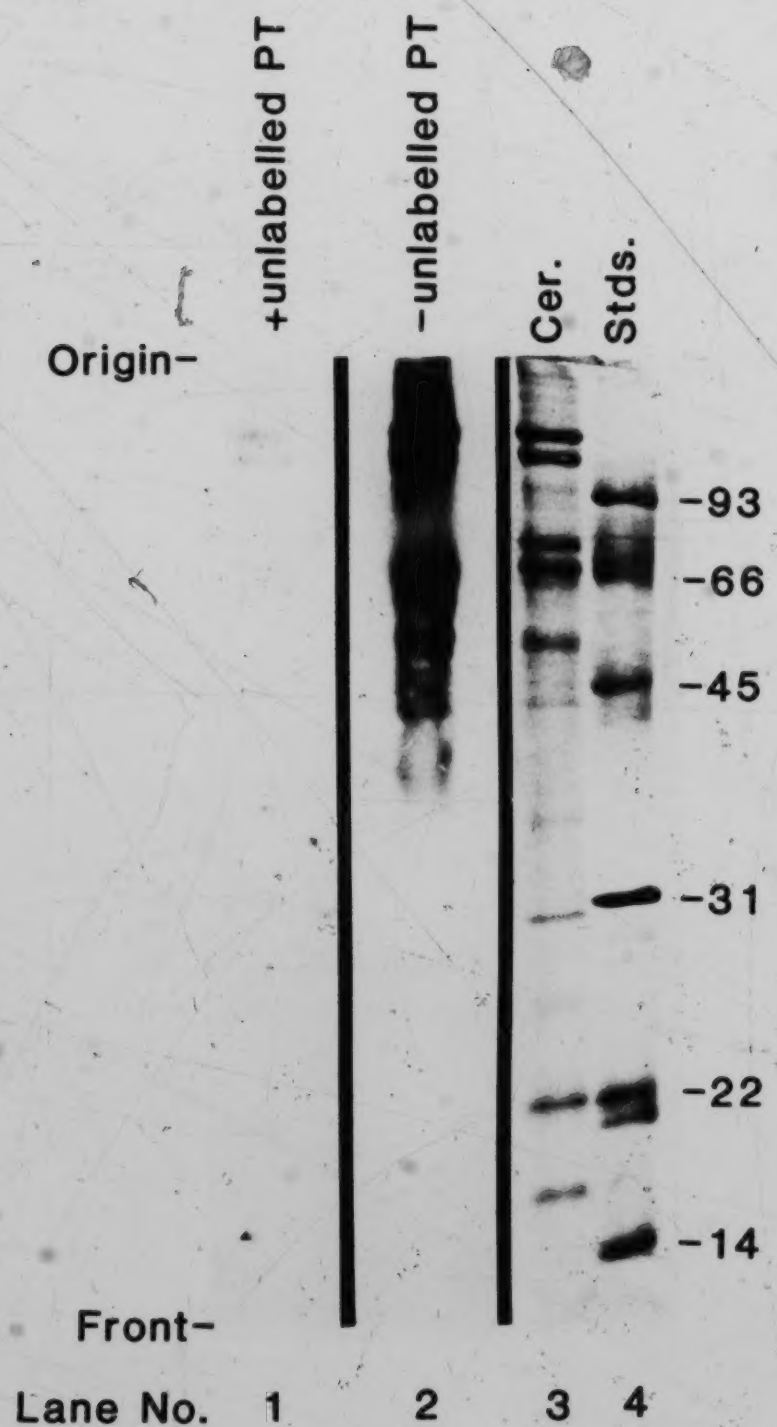

Stds
+2-ME
R/A Cer
Cer
−2-ME
R/A Cer
Cer
+2-ME
R/A Cer
Cer
−2-ME
R/A Cer
Cer
Origin-
97-
66-
45-
31-
22-
14-
Front-
Lane No. 1 2 3 4 5 6
A.
1 2 3 4 5 6
B.

Figure 8. Competition Of Unlabeled Pertussis Toxin (PT) For ^{125}I-PT Binding To Ceruloplasmin On Western Blots. Native ceruloplasmin was separated by discontinuous SDS-PAGE under reducing conditions using a 12.5% separating gel. Following separation, the protein bands were electrophoretically transferred to nitrocellulose sheets, and incubated with a 5% BSA-Tris buffer. One section of the blot was incubated with 0.4 µg of biologically active ^{125}I-PT alone (~100000 cpm), and another section of the blot was incubated with 0.4 µg of ^{125}I-PT and a 2000 fold excess (800 µg) of unlabeled PT. Following overnight incubation, the blots were washed (as described in the Materials and Methods section), air-dried, and autoradiographs of the blots were prepared.


+unlabelled PT
-unlabelled PT
Cer.
Stds.
Origin-
-93
-66
-45
-31
-22
-14
Front-
Lane No. 1 2 3 4

terminal autoproteolytic domain of the ceruloplasmin molecule (figure 10; Takahashi, N., *et al.*, 1984). The section of the blot containing the trypsin/chymotrypsin-treated ceruloplasmin peptides demonstrated no evidence of PT-binding activity (figure 9, panel B, lane 3).

When the PNGase F-treated ceruloplasmin was analyzed by SDS-PAGE (figure 11, lane 3), the banding pattern was different from that of ceruloplasmin incubated in the absence of PNGase F (figure 11, lanes 2 and 4). After treatment with PNGase F, the 138K ceruloplasmin fragment generated two bands, as did the 122K ceruloplasmin fragment, which suggested incomplete deglycosylation of peptides. The ceruloplasmin sample incubated in the absence of PNGase F demonstrated the same banding as native ceruloplasmin. When the PNGase F-treated ceruloplasmin was analyzed by the western blot technique, the deglycosylated ceruloplasmin fragments had very reduced PT-binding activity (figure 11, lane 6), whereas the ceruloplasmin incubated in the absence of PNGase F, maintained PT-binding activity (figure 11, lane 4). As stated above, two bands were generated from each of the 138K and 122K ceruloplasmin fragments. Some PT-binding activity was seen to the upper bands from the 138K and 122K deglycosylated peptides (figure 11, lane 6).

After determining the concentration of protein in the eluate (Lowry assay), the isolated ceruloplasmin fragments (section 2.18) were then separated by discontinuous SDS-

Figure 9. Examination Of PT-binding Activity In Native And Protease-treated Ceruloplasmin Using The Western Blotting Technique. Native ceruloplasmin was incubated with trypsin for 18 h at 37°C. A portion of the trypsinized sample was incubated for an additional 18 h at 37°C with chymotrypsin. The native and enzyme-treated ceruloplasmin samples were then analyzed using discontinuous SDS-PAGE with a 12.5% separating gel and a 5.0% stacking gel. Panel A. Coomassie blue-stained gel of ceruloplasmin incubated at 37°C for 18 h in the absence of proteases (Cer), trypsin treated ceruloplasmin (Trp-Cer) and trypsin/chymotrypsin treated ceruloplasmin (Chymo-Cer). Panel B. Autoradiogram of a western blot of the ceruloplasmin samples shown in Panel A. Acrylamide gels containing native and enzyme-treated ceruloplasmin samples were transferred electrophoretically to nitrocellulose sheets. Following an overnight incubation with sonicated ^{125}I-labeled pertussis toxin, the blots were washed to remove unbound radioactivity, air dried, and autoradiographs of the blots were prepared.

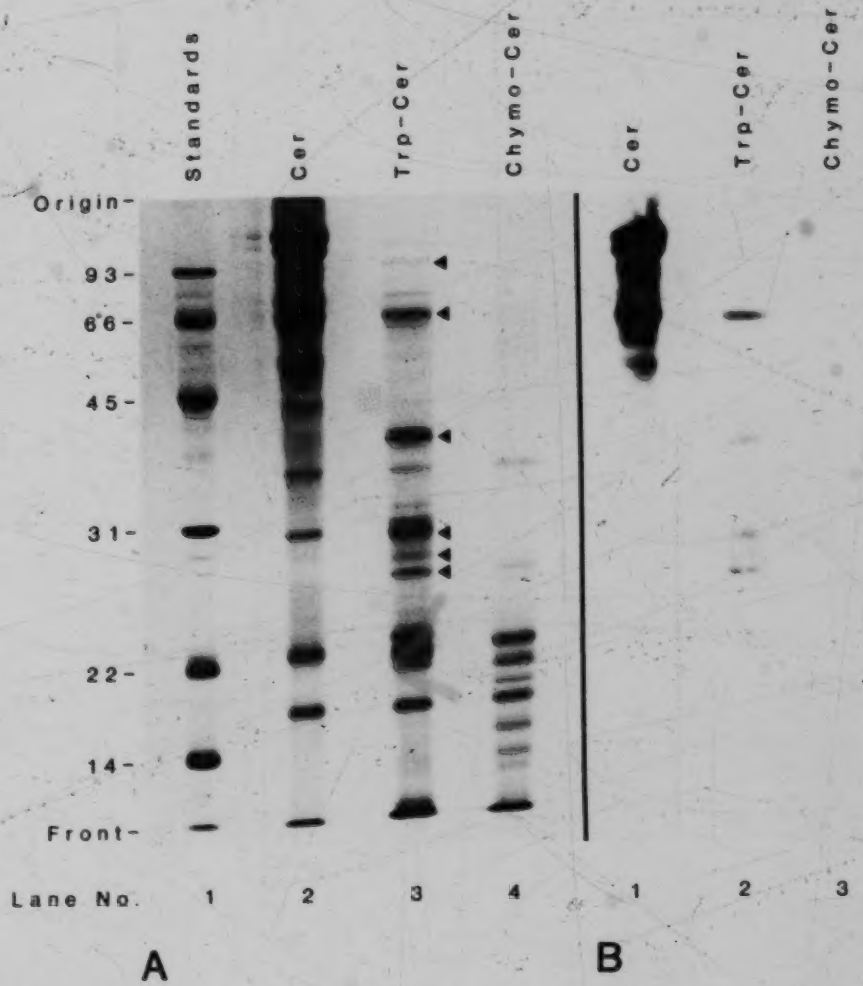
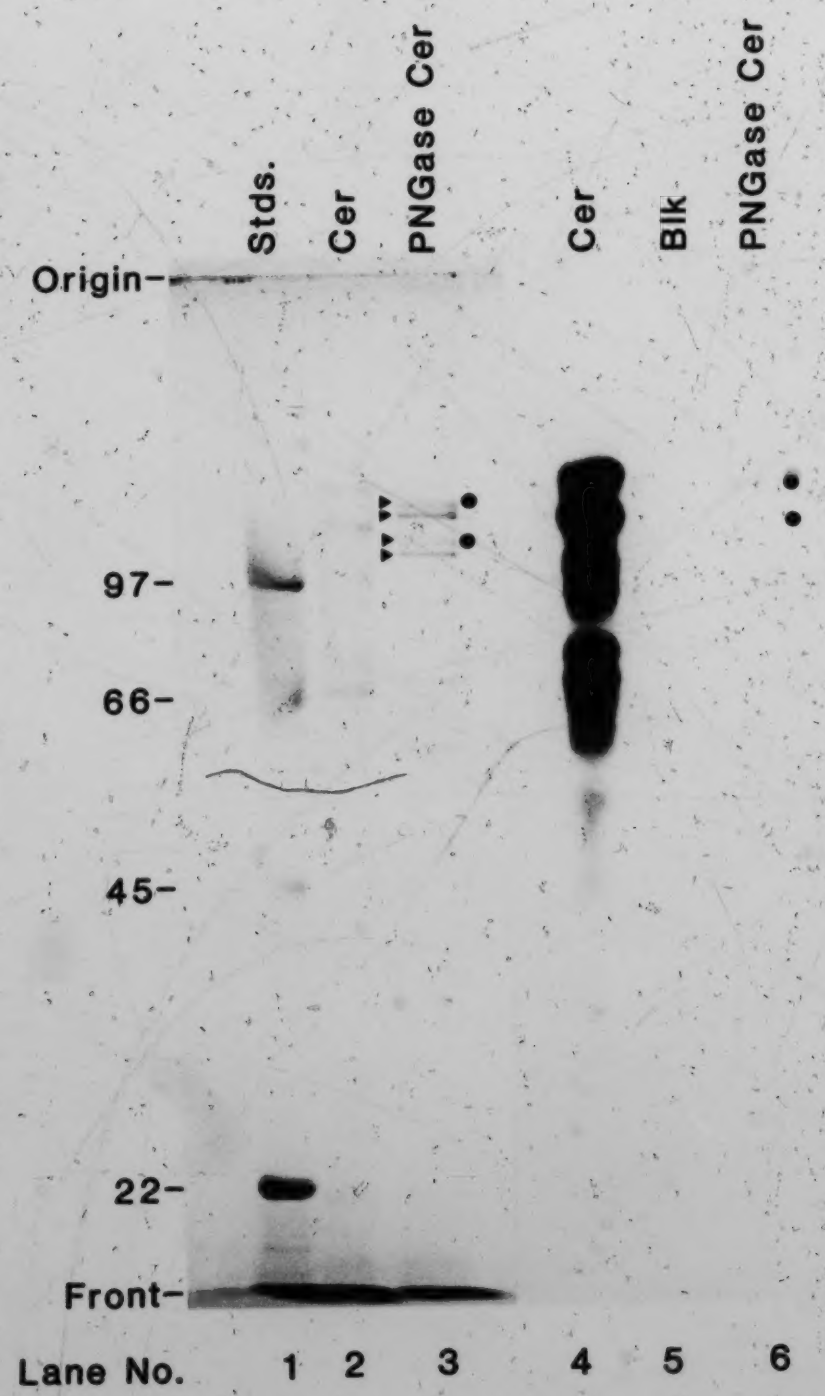

Standards
Cer
Trp-Cer
Chymo-Cer
Cer
Trp-Cer
Chymo-Cer
Origin-
93-
66-
45-
31-
22-
14-
Front-
Lane No. 1 2 3 4 1 2 3
A
B

Figure 10. Amino Terminal Sequence Analysis Of The 72K (Band 4) Fragment.

*(adapted from Takahashi, N., *et al.*, 1984).

Sequence Of The 72K Ceruloplasmin Band	*Published Amino Acid Sequence Of The 67K Ceruloplasmin N-Terminal Domain
K	K
E	E
K	K
H	H
Y	Y
Y	Y
I	I
G	G
I	I
I	I
E	E
T	T

Figure 11. Demonstration Of The Importance Of *N*-linked Oligosaccharides For PT-binding To Ceruloplasmin. The untreated and PNGase F treated ceruloplasmin samples were analyzed by SDS-PAGE using a 10% separating gel, then examined by the western blot procedure as described in the legend to figure 3, using ^{125}I-PT to probe the blots. Lanes 1, 2 and 3 represent the Coomassie blue stained gel of the molecular weight standards (Stds.), ceruloplasmin incubated in the absence of PNGase F (Cer), and PNGase F-treated ceruloplasmin (PNGase Cer) samples respectively. Lanes 4 and 6 represent the autoradiograph of the western blot of the Cer and PNGase Cer samples respectively, following incubation with ^{125}I-PT. Lane 5 is blank. The filled triangles (▶) mark the bands derived from the 138K and 122K ceruloplasmin fragments. The filled circles (●) mark the bands to which residual PT binding activity is found with the PNGase F-treated ceruloplasmin.


Stds.
Cer
PNGase Cer
Cer
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PNGase Cer
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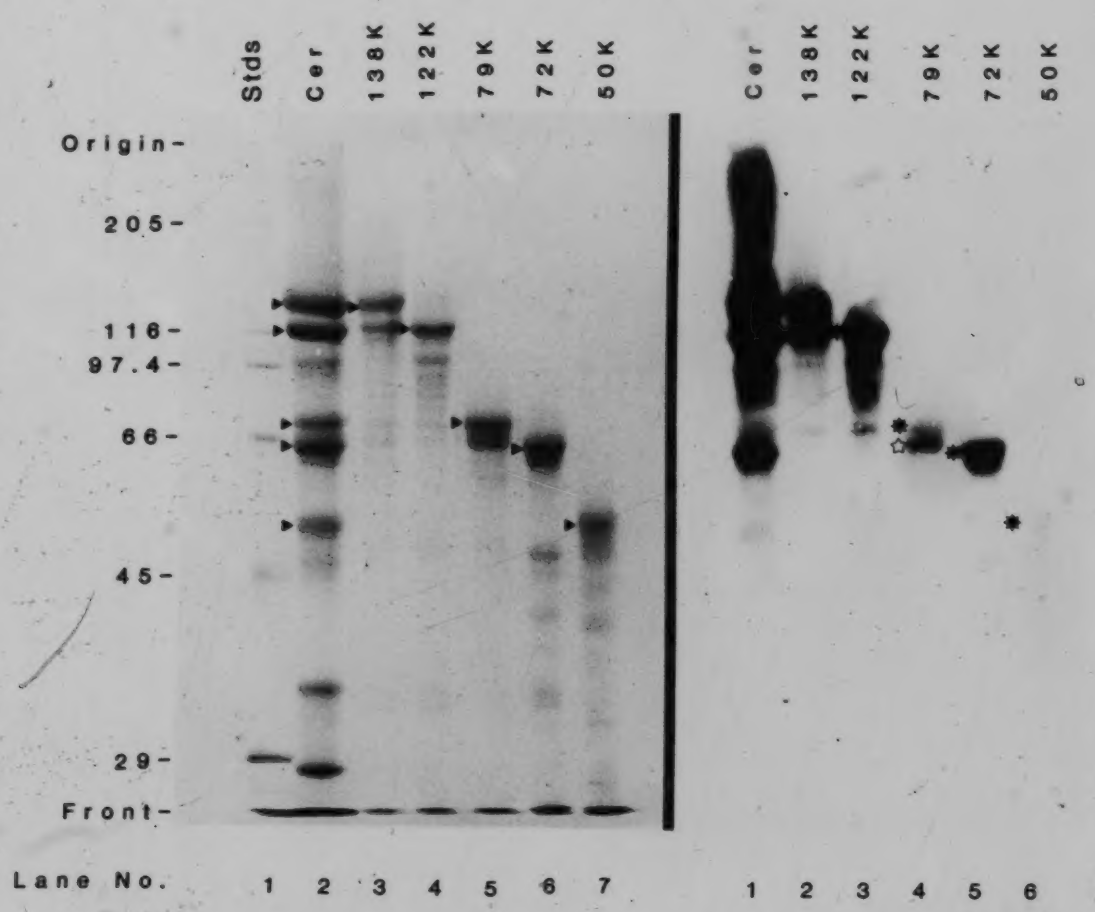
PAGE, and analyzed by the western blot procedure. The ^{125}I-PT demonstrated a high affinity for some of the isolated ceruloplasmin fragments. The ^{125}I-PT bound with high avidity to the 138K (band 1), 122K (band 2), and 72K (band 4) ceruloplasmin fragments, and with a lower avidity to the 79K (band 3), and 50K (band 5), ceruloplasmin fragments (figure 12).

3.5 Fetuin-coated Polystyrene Tube Inhibition Assays.

3.5.1 Native And R/A Ceruloplasmin Inhibition Of ^{125}I-PT And ^{125}I-WGA Binding To Fetuin-coated Tubes.

The polystyrene tube inhibition assay was performed as described in the Experimental Procedures (section 2.14) using native and R/A ceruloplasmin as inhibitors. The IC_{50}'s (concentration of inhibitor resulting in a 50% reduction of PT binding to fetuin) for ^{125}I-PT with native and R/A ceruloplasmin were similar (9.5 ± 3.2 x 10^{-7} M and 1.4 ± 0.7 x 10^{-7} M; figure 13; table 1). The IC_{50}'s for ^{125}I-WGA using native and R/A ceruloplasmin were also very similar (6.2 ± 1.3 x 10^{-7} M and 9.8 ± 0 x 10^{-7} M; figure 13; table 1).

Figure 12. Western Blot Analysis Of Isolated Ceruloplasmin Bands. Following isolation of the individual ceruloplasmin bands from 10% SDS-PAGE gels, the samples were analyzed by electrophoresis and transferred to nitrocellulose sheets. The blot was then incubated with 5% BSA-Tris blocking buffer as described in the Materials and Methods section. The buffer was removed and the blot was incubated overnight at 4°C with ^{125}I-PT in 30 ml of fresh 5% BSA-Tris blocking buffer, washed, and autoradiographs were prepared as described in the Materials and Methods section. Panel A represents the Coomassie-stained gel of molecular weight standards (lane 1), native ceruloplasmin (lane 2), and the isolated ceruloplasmin peptides (lanes 3-6). Panel B represents an autoradiograph of the probed blot of native ceruloplasmin (lane 1), and the isolated ceruloplasmin bands (lanes 2-5). The filled triangles (▶) mark the positions of the individual ceruloplasmin fragments in native ceruloplasmin (lane 2), and with the isolated fragments (lanes 3-7). The filled stars (✸) mark the location of the isolated fragments on the autoradiogram. The unfilled star (☆) on the autoradiogram marks the binding activity of the breakdown product (72K fragment) of the 79K fragment.



A. Coomassie stained B. Autoradiogram

3.5.2 Inhibition Of ^{125}I-PT Binding To Fetuin-coated Tubes By Individual Ceruloplasmin Fragments.

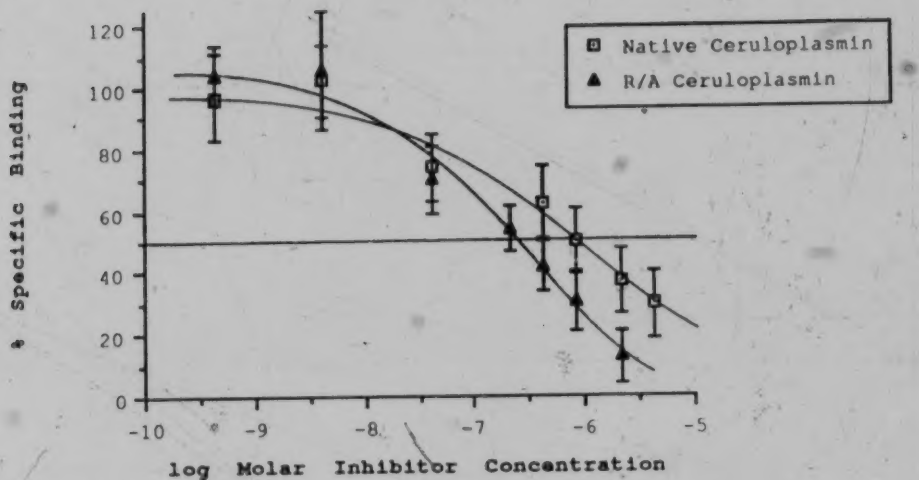
The individual ceruloplasmin fragments were isolated by using the DNS-Cl-labelled ceruloplasmin to identify the relevant bands (sections 2.18 and 3.3). After determining the concentration of protein in the eluate (Lowry assay), the isolated ceruloplasmin fragments were analyzed for PT-binding activity using the fetuin-coated polystyrene tube inhibition assay, as described in the Experimental Procedures (section 2.14). The inhibition of binding of ^{125}I-PT to the fetuin-coated tubes was examined (figure 14). The IC_{50}'s for the individual ceruloplasmin bands are presented in table 2.

3.6 Analysis Of Ceruloplasmin And Ceruloplasmin Fragments By Gel Filtration.

R/A ceruloplasmin, the 72K (67K) fragment, and the 50K ceruloplasmin fragment were analyzed by gel filtration, using a Sephadex G-100 column, with PBS as the running buffer. Based on the gel filtration analysis (figure 15 and table 3), the R/A ceruloplasmin, 72K (67K) and 50K fragments had apparent molecular weights (in solution) that were greater than the molecular weight of the largest protein standard used (aldolase from rabbit muscle, with a molecular weight of 158K). Therefore, these fragments were probably aggregated in solution.

Figure 13. Competitive Binding Inhibition Curves For ^{125}I-labeled Pertussis Toxin (PT) And Wheat Germ Agglutinin (WGA) Using Native And R/A Ceruloplasmin Samples. Varying amounts of inhibitor (native or R/A ceruloplasmin) were added to polystyrene tubes coated with fetuin. After adding ^{125}I-labeled PT or WGA (final concentration was approximately 10^{-9} M), the tubes were incubated overnight. Unbound radioactivity was removed by 4 consecutive washes with 1% BSA in PBS, and the amount of bound radioactivity was recorded. Background binding was determined using a parallel set of polystyrene tubes pre-coated with BSA rather than fetuin, and the maximum (100%) binding in each experiment was measured in fetuin-coated polystyrene tubes containing no inhibitor. Binding at each concentration of inhibitor was performed in triplicate, and the error bars represent the standard error of the mean for each point. The concentration of native or R/A ceruloplasmin giving 50% inhibition of total binding was determined from the graphical analysis of the data.

A. Pertussis Toxin Inhibition Curves



B. Wheat Germ Agglutinin Inhibition Curves

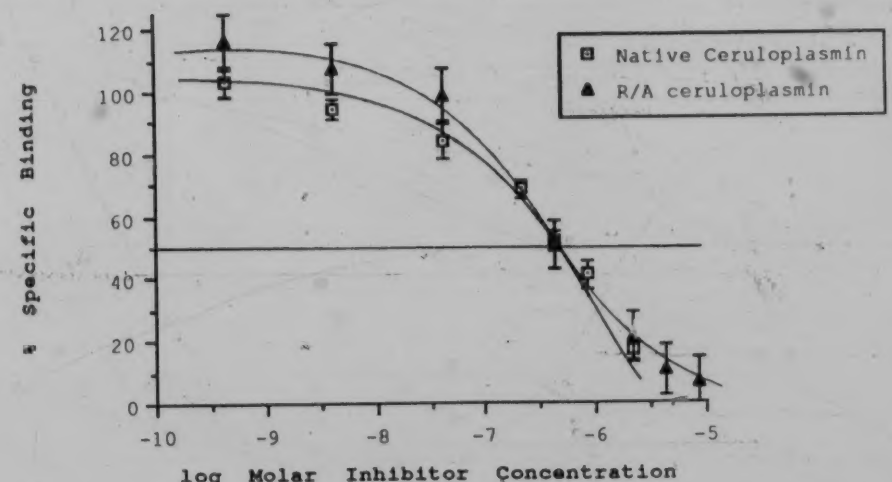


Table 1

Inhibitor	Concentration Resulting in 50% Inhibition of Binding[a]	
	^{125}I-Pertussis toxin	^{125}I-Wheat Germ Agglutinin
Native Ceruloplasmin	$9.5 \pm 3.2 \times 10^{-7}$ M	$6.2 \pm 1.3 \times 10^{-7}$ M
R/A Ceruloplasmin	$1.4 \pm 0.7 \times 10^{-7}$ M	$9.8 \pm 0 \times 10^{-7}$ M
Fetuin*	$9.5 \pm 1.6 \times 10^{-8}$ M	$3.4 \pm 0.2 \times 10^{-6}$ M
Glycophorin A*	$2.1 \pm 0.5 \times 10^{-7}$ M	$4.5 \pm 0.5 \times 10^{-9}$ M
Haptoglobin*	$5.7 \pm 4.5 \times 10^{-7}$ M	$1.6 \pm 0.3 \times 10^{-5}$ M
α1-acid Glycoprotein*	$4.9 \pm 1.4 \times 10^{-6}$ M	$8.6 \pm 1.5 \times 10^{-6}$ M
Transferrin*	$4.3 \pm 0.3 \times 10^{-5}$ M	$>1.1 \pm 0 \times 10^{-4}$ M

[a]Concentrations are the average (± range) of 2 independent determinations. Each independent experiment was performed in triplicate. Concentrations were determined using a molecular weight of 132,000 for ceruloplasmin (native and R/A) (Takahashi *et al.*, 1984), 48,400 for fetuin (Spiro *et al.*, 1960), 16,493 for glycophorin A (Tomita, M., *et al.*, 1978), 100,000 for haptoglobin (Peacock, A.C., *et al.*, 1970), 40,000 for α1 acid glycoprotein, (Schultze, H.E., *et al.*, 1955), and 86,000 for transferrin (Katz, J.H. 1961).

*Previously reported results (Tyrrell, G.J., *et al.*, 1989).

Figure 14. Results Of Competitive Binding Inhibition Experiments For ^{125}I-labeled PT Using Purified Ceruloplasmin Bands. Following isolation, the ceruloplasmin bands were added to polystyrene tubes coated with fetuin, and the maximum binding, percent specific binding and background binding were determined as described in the legend to figure 13. The experiment was performed in triplicate, and the error bars represent the standard error of the mean for each point. The concentration of each ceruloplasmin peptide giving 50% inhibition of total binding was determined from the graphical analysis of the data.

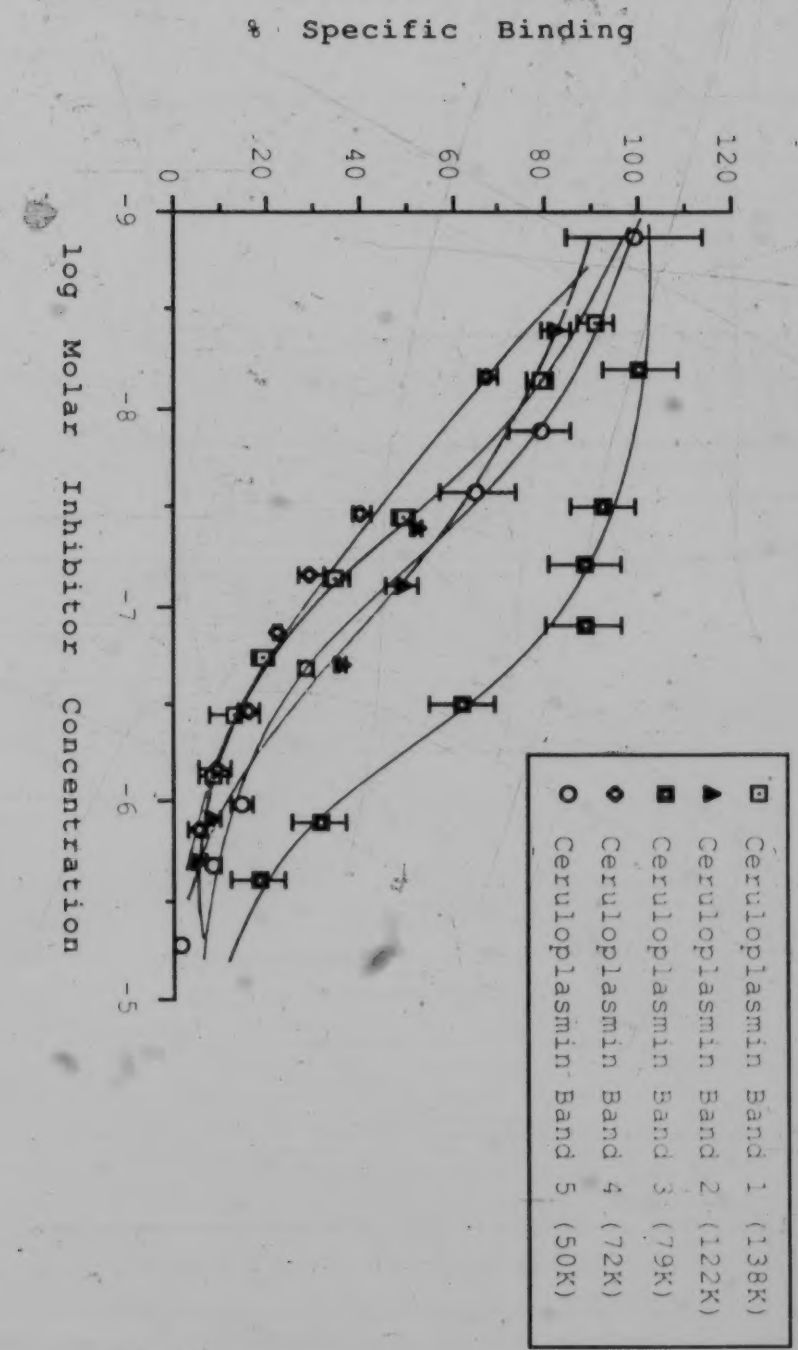

% Specific Binding
0
20
40
60
80
100
120
-9
-8
-7
-6
-5
log Molar Inhibitor Concentration
Ceruloplasmin Band 1 (138K)
Ceruloplasmin Band 2 (122K)
Ceruloplasmin Band 3 (79K)
Ceruloplasmin Band 4 (72K)
Ceruloplasmin Band 5 (50K)

Table 2

Inhibitor	Concentration (± Range) Resulting in 50 % Inhibition of PT Binding[a]
Native Ceruloplasmin[$]	$9.5 \pm 3.2 \times 10^{-6}$ M
R/A Ceruloplasmin[$]	$1.4 \pm 0.7 \times 10^{-7}$ M
PNGase F-Ceruloplasmin*	$>1.1 \pm 0 \times 10^{-4}$ M
Ceruloplasmin Band 1[†] (138K)	$4.8 \pm 0.5 \times 10^{-8}$ M
Ceruloplasmin Band 2[†] (122K)	$9.5 \pm 1.4 \times 10^{-8}$ M
Ceruloplasmin Band 3[†] (79K)	$4.5 \pm 1.9 \times 10^{-7}$ M
Ceruloplasmin Band 4[†] (72K)	$5.8 \pm 4.1 \times 10^{-8}$ M
Ceruloplasmin Band 5[†] (50K)	$9.0 \pm 1.5 \times 10^{-8}$ M

[a]Concentrations are the average (± range) of 2 independent determinations, and each independent experiment was performed in triplicate. [$]Concentration determinations for ceruloplasmin (native and R/A) were performed using a molecular weight of 132,000 (Takahashi, N, *et al.*, 1984). *The PNGase Ceruloplasmin refers to the R/A ceruloplasmin treated with PNGase F; only one concentration was used for the assay. [†]The molecular weights of the individual ceruloplasmin bands were estimated using high molecular weight standards on discontinuous SDS-PAGE linear gel system (7.5-15%), with a 5% stacking gel. Concentration determinations of the inhibiting glycopeptides were performed using the Lowry assay for protein (Lowry, O.H., *et al.*, 1951).

Figure 15. Standardization Of Sephadex G-100 Gel Filtration Column Used To Determine Solution Phase Molecular Weight Of Ceruloplasmin Peptides. Various samples were loaded onto a Sephadex G-100 column (total bed volume of approximately 340 ml) and eluted using PBS. The samples were detected by measuring the absorption of the eluted fractions at 280 nm. After standardization, the column was subsequently used with various ceruloplasmin samples to determine their approximate molecular weight when in solution. The molecular weight standards used were: aldolase from rabbit muscle (158,000), bovine serum albumin (66,000), ovalbumin (45,000), ribonuclease A (13,700), and N-acetyl-L-tyrosine ethyl ester (251).

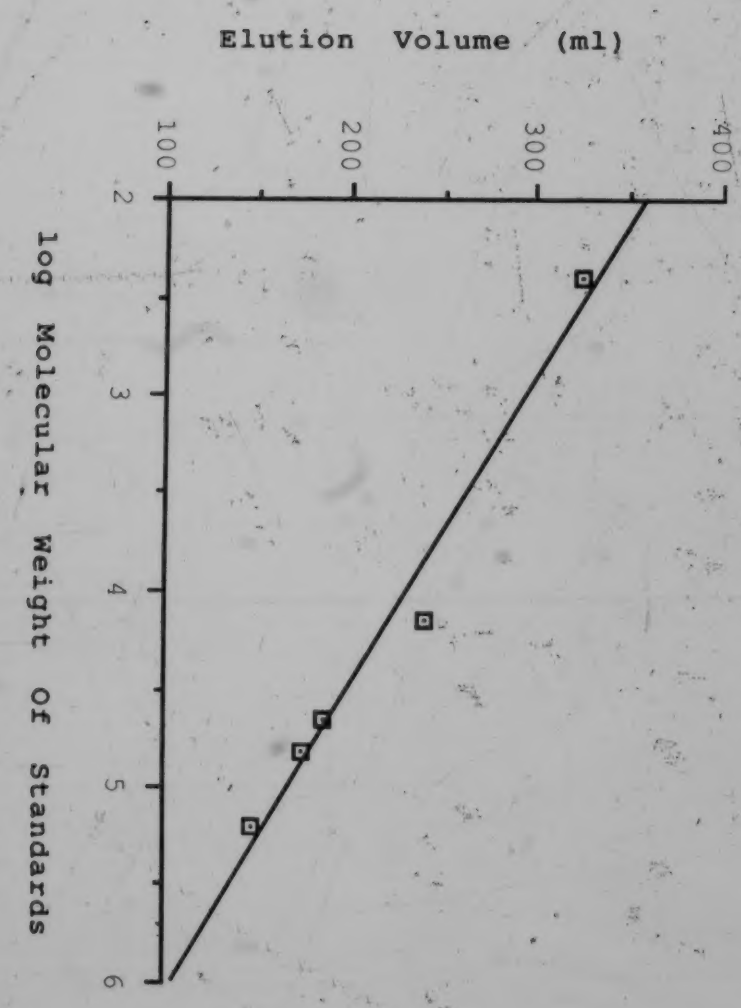

Elution Volume (ml)
100
200
300
400
2
3
4
5
6
log Molecular Weight Of Standards

Table 3

Sample	Molecular Weight As Determined By SDS-PAGE Analysis	Molecular Weight As Determined By Sephadex G-100 Elution Volume*
R/A Ceruloplasmin	138,000	>158,000
72K (67K) Fragment	72,000	>158,000
50K Fragment	50,000	>158,000

*Cumulative data from Sephadex G-100 gel filtration analysis of ceruloplasmin and ceruloplasmin fragments. The molecular weights for the ceruloplasmin fragments were estimated by SDS-PAGE analysis (see figure 4).

4.0 DISCUSSION

Ceruloplasmin is one of several glycoproteins that displays PT-binding activity. Ceruloplasmin inhibits the PT-mediated agglutination of goose erythrocytes, and also demonstrates a high affinity for PT in the fetuin-coated polystyrene tube inhibition assay (figures 13 and 14, and tables 1 and 2). Ceruloplasmin was chosen to study PT's lectin-like properties for three major reasons. (1) It is believed that PT binds only to *N*-linked oligosaccharides, and not to *O*-linked oligosaccharides (linked to serine or threonine) (Sekura, R., and Zhang, Y., 1985; Armstrong G.D., *et al.*, 1988). Ceruloplasmin has only *N*-linked oligosaccharides, for which the sequences are well defined (Yamashita, K. *et al.*, 1981; Endo M., *et al.*, 1982). (2) It is postulated that PT binds with higher affinity to oligosaccharides containing α(2-6)-linked terminal *N*-Acetylneuraminic Acid (NeuAc) residues, and with lower affinity to α(2-3)-linked terminal NeuAc residues (Armstrong, G.D., *et al.*, 1988; Tyrrell, G.J., *et al.*, 1989; Heerze, L.D., and Armstrong, G.D., 1990). Ceruloplasmin is primarily glycosylated with α(2-6)-linked terminal NeuAc residues (figure 3; Yamashita, K., *et al.*, 1981; Endo M., *et al.*, 1982). (3) The sequences and linkage of the other terminal carbohydrates of ceruloplasmin [αNeuAc(2-6)ßGal(1-4)GlcNAc] are identical to those carbohydrates demonstrated to be important in PT-

fetuin binding interactions (Armstrong, G.D., *et al.*, 1988).

My initial investigations were focused on examining the structural components of the ceruloplasmin oligosaccharides that were required for optimal PT binding, in addition to investigating the functional importance of the peptide portion of the ceruloplasmin molecule. Because PT behaves as a lectin, it was hoped that by comparing the binding activity of PT with a lectin of known binding specificity, more information about the binding specificity of PT could be derived. I chose wheat germ agglutinin (WGA) for this purpose for two major reasons: first, it is known that WGA binds to oligosaccharides terminating in α(2-3)-linked NeuAc, in preference to those oligosaccharide chains with terminal α(2-6)-linked NeuAc groups (Kronis, K.A., and Carver, J.P., 1985; Furukawa, K.A., *et al.*, 1986). Second, the binding interactions of PT and WGA with several glycoproteins have been previously investigated (Tyrrell, G.J., *et al.*, 1989). However, as was recently demonstrated by Tyrrell, *et al.* (1989), WGA also strongly interacted with glycoproteins containing predominantly α(2-6)-linked terminal sialic acid residues. Thus, although WGA may preferentially bind to terminal α(2-3)-linked NeuAc residues, the findings supported the suggestion that the stringency of this interaction is not absolute, and sialoglycopeptides with terminal α(2-6)-linked NeuAc residues are also capable of strong binding interactions

with WGA (Kronis, K.A., and Carver, J.P., 1985; Furukawa, K.A., *et al.*, 1986).

I first compared the binding of PT and WGA to ceruloplasmin using the fetuin-coated polystyrene tube inhibition assay. The results demonstrated that PT was capable of strongly interacting with ceruloplasmin in that ceruloplasmin inhibited the binding of PT to fetuin at μM concentrations (figures 13 and 14, and tables 1 and 2). In addition, as was also reported previously with other glycoproteins (Tyrrell, G.J., *et al.*, 1989), differential binding of PT and WGA to ceruloplasmin was seen. That is, the binding of PT and WGA to ceruloplasmin was similar, but not identical. Thus, although the lectins may recognize similar sugar sequences, dissimilarities between the structures of PT and WGA may confer differential binding.

Further analysis of the results from the polystyrene tube inhibition studies revealed that native ceruloplasmin inhibited the binding of ^{125}I-PT and ^{125}I-WGA to fetuin at concentrations similar to those reported for fetuin itself (figure 13 and table 1; Tyrrell, G.J., *et al.*, 1989). The concentration of inhibitor resulting in a 50% reduction of PT binding activity (IC_{50}) for ceruloplasmin-WGA binding interactions was low (ie. displayed inhibitory activity at low concentration), confirming that WGA was capable of strongly interacting with glycoproteins with (almost exclusively) α(2-6)-linked terminal sialic acid residues. These findings support the view that WGA is capable of high

avidity interactions with sialoglycopeptides with terminal α(2-6)-linked NeuAc residues.

Next, to examine the functional importance of the peptide portion of the molecule, ceruloplasmin was irreversibly reduced and alkylated to cleave disulfide bonds. The binding of the R/A ceruloplasmin to PT and WGA was then examined using the fetuin-coated polystyrene tube inhibition assay. The results of these investigations demonstrated that reduction and alkylation of disulfide bonds in ceruloplasmin had little affect on its ability to inhibit the binding of PT or WGA to fetuin, in that the inhibitory activity of R/A ceruloplasmin was similar to that of the native molecule. These findings implied that the integrity of disulfide bonds was not required for optimal interactions of PT with ceruloplasmin.

To further examine the functional importance of the peptide portion of the molecule, native ceruloplasmin was subjected to limited proteolysis using trypsin and chymotrypsin. As was reported previously for fetuin (Armstrong, G.D., *et al.*, 1988), proteolysis dramatically reduced the ability of ceruloplasmin to inhibit the agglutination of goose erythrocytes (Armstrong, G.D., unpublished data). This inhibitory activity declined by a factor of eight after the ceruloplasmin had been partially digested with trypsin. Subsequent digestion of the trypsin-treated ceruloplasmin with chymotrypsin resulted in the hemagglutination inhibition (HAI) activity further

decreasing almost 18 fold. These observations support earlier theories that the number (valency) of oligosaccharide units on glycoprotein receptors, or structural components of the peptide sequences may also contribute to binding activity (Armstrong, G.D., *et al.*, 1988).

The autoproteolytic activity of ceruloplasmin has been the subject of many investigations (Dwulet, F.E., and Putnam, F.W. 1981; Prozorovski, V.N., *et al.*, 1982; Takahashi, N., *et al.*, 1983; Ortel, T.L., *et al.*, 1984). When the ceruloplasmin samples used for our investigations were analyzed by SDS-PAGE, several bands were detected (figure 4) due to the autoproteolytic activity. The major ceruloplasmin bands had estimated molecular weights (SDS-PAGE analysis) of 138K, 122K, 79K, 72K, 50K, 36K, 30K, 22K, and 18K (figure 4). There were also minor ceruloplasmin bands at 95K and 90K.

The next step in my investigation was to identify autoproteolytic ceruloplasmin fragments with PT-binding activity by affinity chromatography. For this purpose, a small column of PT immobilized onto Sepharose (agarose) was prepared (see section 2.4). It was hoped that only those ceruloplasmin fragments with PT-binding activity would bind to the immobilized PT and could be subsequently eluted from the PT-Sepharose. To identify ceruloplasmin peptides interacting non-specifically with the Sepharose, a fetuin-agarose column was used. When native ceruloplasmin was

passed through the fetuin-agarose control column, silver stained SDS-polyacrylamide analysis of the bound material revealed a 30K peptide (figure 5, top panel). In contrast, when native ceruloplasmin was passed through a PT-agarose column, the 138K, 122K, 79K, 72K, 50K, 30K, and 22K peptides were all detected in the fractions that were eluted with diethanolamine (figure 5, lower panel). The 18K peptide, however, was not found in the diethanolamine fractions from either the PT-agarose or fetuin-agarose columns, though it was seen in the wash fractions. Thus, it was evident that the 30K peptide was interacting with the agarose non-specifically, and that the 18K band was not interacting with the PT or fetuin-agarose. However, the results proved inconclusive as to precisely which of the other ceruloplasmin fragments were specifically interacting with PT. For example, it was unknown if the lower molecular weight fragments (<138K) of intact ceruloplasmin seen on SDS-PAGE gels of the diethanolamine fractions were binding directly to PT-Sepharose, or were merely attached by disulfide bonds or non-covalent intramolecular interactions to those fragments containing PT-binding activity.

To determine if disulfide bonds had a role in the binding of some of the ceruloplasmin peptides to PT-agarose, the R/A ceruloplasmin preparation which had been shown to retain its PT-binding activity was passed through the PT-agarose column. The silver-stained SDS-

polyacrylamide gels of the PT-Sepharose-bound R/A ceruloplasmin peptides revealed an identical banding pattern to those gels containing PT-Sepharose-bound native ceruloplasmin. Thus, reduction and alkylation of disulfide bonds had no affect on the binding of ceruloplasmin to PT-Sepharose. Therefore, it was still unclear which fragments from the eluted fractions seen on the gels were specifically interacting with the PT.

To circumvent the difficulties associated with the affinity isolation technique, I adopted the western blotting procedure (Burnette, W.N., 1981) to identify ceruloplasmin peptides with PT binding activity. Prior to analysis by the western blot procedure, the native and R/A ceruloplasmin samples were separated by SDS-PAGE in the presence and absence of 2-mercaptoethanol. After staining the gel with Coomassie blue, it was demonstrated that there was no difference in the banding pattern of the R/A ceruloplasmin regardless of whether or not 2-mercaptoethanol was used during sample preparation. This observation confirmed that the reduction and alkylation reaction had gone to completion (figure 7, panel A, lanes 3 and 6). As expected, the banding pattern of the native ceruloplasmin was different when 2-mercaptoethanol was omitted from the sample buffer prior to analysis by SDS-PAGE. Higher molecular weight aggregates consisting of sulfhydryl-linked forms of the native ceruloplasmin were seen (figure 7, panel A, lane 6).

Immediately following electrophoresis, the native and R/A ceruloplasmin peptides were transferred from the SDS-PAGE gels to nitrocellulose sheets, and the ability of the peptides to interact with PT was examined by incubating the blots with ^{125}I-PT. Autoradiograms of the resulting blots revealed that all of the low molecular weight ceruloplasmin peptides were unable to bind to PT. All peptides seen on the autoradiograms had molecular weights of >50K, and none of the low molecular weight peptides seen on SDS-PAGE gels displayed any appreciable PT-binding activity (figure 7, panel B).

Since this work represented the first evidence that that ^{125}I-PT prepared as described by Armstrong and Peppler (1987), could be used directly as a probe for receptors on western blots, it was also necessary to demonstrate the specificity of the interaction of ^{125}I-PT and ceruloplasmin in the western blotting procedure. The specificity of the interaction was evaluated by preparing replicate blots of native ceruloplasmin (from an SDS-PAGE gel). Both blots were incubated with ^{125}I-PT (0.4 µg), however one blot was incubated in the presence of excess (800 µg) unlabeled PT. As expected, the unlabeled PT competed with the ^{125}I-PT for the ceruloplasmin binding sites (figure 8). This observation provided additional evidence that binding activity of PT is maintained when it is iodinated in the presence of immobilized fetuin (Armstrong, G.D., and Peppler, M.S., 1987).

An interesting observation on the western blots of native and R/A ceruloplasmin peptides (figure 7, panel B, lanes 5 and 6) was the apparent increased binding of ^{125}I-labeled PT to both native and R/A ceruloplasmin samples that had not been treated with 2-mercaptoethanol. Although the reason for this observation is not clear at this time, Hackstadt *et al.*, (1986) observed that HeLa cell membranes failed to bind to a 30K Chlamydial outer membrane cell attachment protein in western blots of mercaptoethanol-treated samples. This effect however, was not observed if, prior to SDS-PAGE, the Chlamydial outer membranes were dissolved in SDS sample buffer containing dithiothreitol instead of 2-mercaptoethanol. Therefore, in the Chlamydia receptor studies, the affect appeared to be related to the use of 2-mercaptoethanol rather than reduction of disulfide bonds *per se*. It is possible that the effects we observed in PT-probed western blots of ceruloplasmin are also related to a non-specific effect of 2-mercaptoethanol.

The previous western blot analysis had demonstrated that PT was only capable of interacting with high molecular weight ceruloplasmin fragments and not to low molecular weight fragments. However, before drawing any firm conclusions regarding PT binding to ceruloplasmin peptides, it was important to ensure that complete transfer of all high and low molecular weight ceruloplasmin peptides to the nitrocellulose sheets had occurred. For this purpose, a duplicate blot was incubated with the amido-black stain.

Results from this procedure demonstrated that all of the ceruloplasmin fragments seen in Coomassie-stained gels were efficiently transferred to nitrocellulose (figure 7, panel A). In addition, following peptide transfer, gels were routinely stained with Coomassie blue to ensure that the majority of peptides had been extracted from the gel. Therefore, the observation that PT did not bind to the low molecular weight ceruloplasmin fragments was not related to incomplete transfer of these fragments to the nitrocellulose sheets.

In an attempt to identify the minimum structure of ceruloplasmin with strong PT binding activity, I proceeded to use the western blot procedure to identify PT-binding ceruloplasmin peptides generated by limited proteolysis with trypsin and chymotrypsin. The SDS-PAGE banding pattern of the trypsin digest revealed that most of the high molecular weight ceruloplasmin peptides had been cleaved to lower molecular weight peptides (figure 9, panel A, lane 3). More specifically, the 138K and 122K fragments almost completely disappeared as lower molecular weight peptides were generated. As expected, the banding pattern of the trypsin and trypsin/chymotrypsin digest on SDS-PAGE revealed the presence of a large number of low molecular weight fragments, the former being less degraded than the latter (figure 9, panel A, lane 4).

As mentioned previously, proteolysis dramatically affected the ability of ceruloplasmin to interact with PT

as judged by HAI (Armstrong, G.D., unpublished data). Nonetheless, I postulated that low molecular weight ceruloplasmin peptides with PT-binding activity could be identified if limited proteolysis was used to generate the fragments. When a western blot of these digests was prepared, the trypsin/chymotrypsin-treated ceruloplasmin peptides contained no evidence of PT-binding activity (figure 9, panel B, lane 3). However, the trypsin digest contained peptides with reduced PT-binding activity; ceruloplasmin bands with apparent molecular weights of 72K, 39K, 30K, 28K, 26K and a weakly interacting band at 122K were seen on autoradiograms of nitrocellulose blots (figure 9, panel B, lane 2). Amino terminal sequence analysis of the band at 72K demonstrated that this peptide was probably identical to the 67K autoproteolytic domain of the ceruloplasmin molecule (figures 2 and 10; Takahashi, N., *et al.*, 1984). The ability of PT to interact with the lower molecular weight peptides (39K-26K) generated from the proteolysis of high molecular weight fragments, was an interesting observation. In the prior western blots of native and R/A ceruloplasmin (figure 7), no PT-binding activity was seen with any of the low molecular weight peptides (<50K) that were derived from autoproteolysis. The binding of PT to these low molecular weight peptides derived from proteolysis of ceruloplasmin with trypsin indirectly supported my earlier hypothesis that low molecular peptides derived from autoproteolysis of native

ceruloplasmin were not seen on autoradiograms of ^{125}I-PT probed western blots, because they did not interact with PT. That is, the low molecular weight native ceruloplasmin fragments seen on SDS-PAGE gels were present on nitrocellulose blots as confirmed by the staining of blot of native ceruloplasmin peptides with amido black (figure 7; panel A), but did not interact with PT.

To confirm that PT-oligosaccharide interactions were the major component in PT-glycoprotein binding in western blots, as had been suggested in prior studies (Capiau, C., *et al.*, 1986; Armstrong, G.D., *et al.*, 1988; Brennan, M.M., *et al.*, 1988), I attempted to use a stain specific for carbohydrates, to determine whether the ceruloplasmin peptides with PT-binding activity on western blots were, in fact, glycosylated. In addition, I wanted to demonstrate that peptides displaying intense PT-binding activity on western blots had a higher degree of glycosylation than peptides with low PT-binding activity (ie. the intensity of the bands was related to the affinity of PT-binding in solution). The Boehringer Mannheim Glycan Detection Kit was chosen for this purpose initially because of its high sensitivity (0.1-10 µg of glycopeptide) and its applicability to western blots. The assay involves mild periodate oxidation of hydroxyl groups to aldehyde groups. The spacer linker digoxigenin is then covalently attached to these aldehydes via a hydrazide group. Digoxigenin labelled glycoconjugates are subsequently detected in an

enzyme immunoassay using an antibody (alkaline phosphatase conjugate) with a specificity for digoxigen. However, a major limitation of this staining technique was that the detection limit varies for different glycoproteins, depending on the content of an individual carbohydrate. Thus, because all carbohydrates do not stain equally (as stated in the manufacturers instructions), I felt that the results may have been misleading in that it might be unknown if a band that stained intensely had a high degree of glycosylation, or simply contained carbohydrates that were more sensitive to the stain. Other problems encountered with this staining technique were the high degree of background staining of regions not containing protein bands, and the fact that the ceruloplasmin peptides tended to lift off the nitrocellulose sheets during the final staining procedure. In light of these problems, and after several unsuccessful attempts to stain blots of ceruloplasmin peptides, I abandoned the approach.

As an alternative, I decided to remove the *N*-linked oligosaccharides of ceruloplasmin enzymatically, and test the deglycosylated peptides for PT-binding activity using the western blot technique. For this purpose, R/A ceruloplasmin was incubated with PNGase F (Glycopeptide-*N*-glycosidase) from *Flavobacterium meningosepticum*, which specifically removes biantennary, triantennary, and tetraantennary *N*-linked oligosaccharides from glycopeptides (Tarentino, A.L., *et al.*, 1985). The cleavage occurs

between the innermost residue of N,N'-diacetylchitobiose and the asparagine to which the oligosaccharide is linked. I found that the ceruloplasmin peptides incubated in the presence of PNGase migrated faster than those ceruloplasmin peptides incubated in the absence of PNGase F when analyzed by SDS-PAGE; indicating that the treated peptides had lower molecular weights than the native peptides, and that the *N*-linked oligosaccharide chains had been removed. Importantly, it was apparent that the peptides were not completely deglycosylated. A comparison of the SDS-PAGE banding patterns of ceruloplasmin incubated in the presence and absence of PNGase F-treated ceruloplasmin (figure 11, lanes 2 and 3 respectively) demonstrated that two bands were generated from each of the 138K and 122K ceruloplasmin bands (figure 11, lane 3), which suggested incomplete removal of *N*-linked oligosaccharide chains from these bands. We also expected from previous studies (Armstrong, G.D., *et al.*, 1988; Brennan, M.M., *et al.*, 1988) that by removing the *N*-linked sugars, PT-binding activity would be completely abolished. The autoradiogram of the blot demonstrated that PT-binding activity of the PNGase F-treated ceruloplasmin was almost completely abolished. A small degree of binding remained in two of the high molecular weight bands, that retained some of their oligosaccharide groups (figure 11; lane 6). It was also demonstrated that the ceruloplasmin incubated in the presence of PNGase F was unable to inhibit the binding of

^{125}I-PT in the polystyrene tube assay (table 2). It is important to note that because ceruloplasmin does not contain *O*-linked oligosaccharides, their importance to PT binding could not be evaluated. Ideally, a glycoprotein such as fetuin with both *N*-linked and *O*-linked oligosaccharide chains could be subjected to treatment with PNGase F, and the abolishment of PT binding by the removal of the *N*-linked sugars would confirm the specificity of PT for *N*-linked oligosaccharide chains. It is important to note that although *N*- and *O*-linked oligosaccharides contain similar carbohydrates, they differ in sequence of the individual sugars. In addition, they differ in the type of the glycosidic linkage to the peptide backbone (Nathan, S., 1975).

It was clear that the carbohydrate moiety of ceruloplasmin was of primary importance for PT-binding activity. However the number (valency) of oligosaccharide chains required for optimal PT binding was unknown. Previous studies have demonstrated that PT is able to bind to glycophorin A, which only contains one *N*-linked oligosaccharide chain (Tomita, M., *et al.*, 1978; Yoshima, H., *et al.*, 1980), and transferrin which contains two *N*-linked oligosaccharide chains (Spik, G. 1975), although transferrin binds PT at a much lower affinity than fetuin and glycophorin (Tyrrell, G.J., *et al.*, 1989). However, as mentioned earlier, these glycoproteins were not good models for examining the valency question.

Using ceruloplasmin as a model for PT-glycoprotein interactions, we had hoped to gain further insight into the valency required for optimal PT binding. As stated earlier, the 72K (67K) fragment of ceruloplasmin contains three of the molecule's four *N*-linked oligosaccharide chains (Takahashi, N., *et al.*, 1984). It was unknown if the fourth oligosaccharide unit on the 50K fragment was contributing to PT-binding, or if the three oligosaccharide chains on the 72K (67K) fragment were sufficient for optimal PT-binding activity. We reasoned that by determining the binding activity of the various ceruloplasmin peptides, we could establish if the binding of the 72K (67K) ceruloplasmin fragment was comparable to that of native ceruloplasmin, and thus the valency of the 72K (67K) fragment (three *N*-linked oligosaccharide chains) was sufficient for optimal PT binding.

Although the western blot procedure was instrumental in identifying native, R/A, and enzymatically-treated ceruloplasmin peptides with PT-binding activity, an important limitation of the technique was that the relative affinities of those peptides seen on autoradiograms could not be readily assessed. Also, though the amount of protein added to a particular SDS-PAGE gel was known, the concentration of protein in any of the individual bands was not known with certainty. For these reasons, I felt that it was necessary to isolate the individual ceruloplasmin fragments with PT-binding activity on western blots, and

determine their IC_{50}'s in the fetuin-coated polystyrene tube assay.

Because of the strong intramolecular attractions and the autoproteolytic nature of ceruloplasmin, we postulated that isolation of the high molecular weight fragments would be difficult. Prior attempts (Ortel, T.L., *et al.*, 1983) to isolate individual ceruloplasmin fragments by HPLC were not possible without digestion of ceruloplasmin with trypsin to degrade the high molecular weight (138K-79K) peptides. Even after limited proteolytic cleavage, strong intramolecular interactions exist between the individual fragments, causing them to aggregate. The presence of these strong intramolecular interactions was confirmed by our attempts to isolate PT binding fragments from ceruloplasmin by affinity chromatography.

Following proteolysis, it is possible to isolate the stable 72K (67K) fragment (by gel filtration HPLC using 8 M urea as the running buffer; Ortel, T.L., *et al.*, 1983). However, because isolation of high molecular weight (>72K) ceruloplasmin peptides was important (to examine their PT-binding activity), HPLC was of little use. Instead, I attempted to isolate the individual bands from fixed and stained SDS-PAGE gels.

It was soon discovered, unfortunately, that the routine fixing and staining process caused severe degradation of the high molecular weight peptides, and when the isolated samples were re-examined by SDS-PAGE, no

intact high molecular weight peptides were visible. I then found that a combination of two procedures could be used to identify ceruloplasmin peptides in unfixed, unstained gels.

The first technique involved the use of Dansyl chloride (DNS-Cl) to label the ceruloplasmin peptides. The dansylated peptides could then be mixed with native ceruloplasmin peptides, and the mixture could be analyzed by SDS-PAGE. The separated peptides could then be visualized without fixing and staining the gel. Though the protocol for labelling proteins in this manner suggested boiling the samples with the DNS-Cl (Talbot, D.N., and Yphantis, D.A., 1971), we found that dansylation could only be accomplished if the ceruloplasmin-DNS-Cl solution was left at room temperature (section 2.18), as is the protocol for DNS-Cl labelling of amino acids (Allen, G., 1981).

Following incubation at room temperature for one week, the DNS-ceruloplasmin samples were mixed with native ceruloplasmin and separated by SDS-PAGE. Immediately following electrophoresis, the unfixed, unstained gels could be placed on an ultraviolet lightbox, and the DNS-ceruloplasmin peptides could be visualized by their fluorescence. The individual bands could then be readily cut out from the gels, crushed into very small pieces, and placed into a test tube with Tris-SDS elution buffer. Following overnight incubation, the ceruloplasmin peptides were then collected in the supernatant solution after centrifugation. A portion of the supernatant solution was

again subjected to SDS-PAGE, to confirm sample purity (figure 12).

When using fixed and stained gels, the Coomassie blue (non-covalently attached to the peptides), drastically interfered with assays for determination of protein concentration. In this respect, the dansylation procedure proved of great utility in that the DNS-Cl absorbs in the ultraviolet range (190-380 nm), whereas the Bradford and Lowry assays, for determination of protein concentration (Bradford, M.M., 1976; Lowry, O.H., *et al.*, 1951), absorb in the visible spectrum (380-780 nm), thus the DNS-Cl did not interfere with either assay.

The second protocol I adopted was identical to the above procedure, however, instead of placing the gel on a ultraviolet (μv) light box following electrophoresis (for visualization of the separated fragments), the gel was immediately placed in an ice-cold solution of 0.25 M KCl for 15 min, and then briefly washed with cold water. The individual bands appeared as a faint white precipitate in the gel (Hager, D.A., and Burgess, R., 1980). The reason for using this procedure in combination with the DNS-Cl protocol, was that excess DNS-Cl sometimes produced a smear (figure 6, panel A, lane 1) which obscured the resolution of the individual bands, which were in close proximity to each other. When used in combination, these procedures allowed for the separation of the intact ceruloplasmin peptides (figure 12, panel A), which could then be used in

the polystyrene tube inhibition assay (figure 14 and table 2), and the western blot procedure (figure 12, panel B).

The isolated ceruloplasmin bands demonstrated binding activity similar to that of native ceruloplasmin in the polystyrene tube inhibition assay. It is important to note that the molecular weights of the ceruloplasmin fragments were estimated relative to protein standards on a 7.5% to 15% linear gel system (figure 4). The estimates of the molecular weights were used to determine the concentration of ceruloplasmin fragments in the polystyrene tube inhibition assay. Bands 1, 2, 3, 4, and 5 of ceruloplasmin had molecular weights of 138K, 122K, 79K, 72K, and 50K respectively. These molecular weight estimates were used in the peptide fragment calculations to be consistent with all of the concentration determinations of the individual fragments.

It was demonstrated that the isolated 72K (67K) fragment (band 4) inhibited the binding of PT to the immobilized fetuin as well as the 138K (band 1) and 122K (band 2) ceruloplasmin bands of native ceruloplasmin (table 2). The 72K (67K) peptide contains three oligosaccharide chains (figures 2 and 3; Takahashi, N., *et al.*, 1984). This, in combination with the PNGase F results, initially suggested that a valency of three oligosaccharide chains was sufficient for optimal PT-binding activity.

On western blots of native ceruloplasmin, the 79K and 50K ceruloplasmin fragments (bands 3 and 5 respectively)

demonstrate a markedly lower affinity for PT, in comparison to bands 1, 2, and 4. However, as mentioned previously, the results obtained from the western blot procedure were of limited applicability because of the inability to assess the avidity of the PT-receptor interaction with this procedure. In addition, the concentration of the particular fragment on the blot was unknown; although a known amount of protein was applied to the gel, the concentration of each individual breakdown product (fragment) was unknown. To circumvent this problem, the avidity of the interactions were assessed using the fetuin-coated polystyrene tube inhibition assay. The results indicated that, in comparison to the other fragments (bands 1, 2 and 4), the 79K fragment had a slightly lower affinity for PT (figure 14 and table 2). The reason for the apparent lower affinity of the PT-band 3 interaction is unknown, though it is possible that a peptide fragment is causing a steric hindrance. This conclusion is based on the fact that a breakdown product of the 79K fragment is the 72K fragment, which strongly interacts with PT in both the western blot assay and the polystyrene tube assay. In addition, further analysis of the breakdown products from the high molecular weight ceruloplasmin fragments (138K and 122K fragments, figure 12, panel A) yields valuable information about the purity of the ceruloplasmin preparation. It is apparent that the 122K, 79K, and 72K fragments are generated by the breakdown of the 138K

fragment. This conclusion is based on the finding that all of these peptides are seen after autoproteolysis of the isolated 138K fragment (figure 12, lane 3). In addition, the 79K and 72K peptides are also seen as products from the autoproteolysis of the isolated 122K fragment (figure 12, lane 4). Further, the 72K fragment is also seen as a product of the autoproteolysis of the 79K fragment. Since the amino acid sequence analysis of the 72K fragment clearly demonstrates that the peptide originates from the N-terminus of ceruloplasmin, it is inherent that the other fragments also originated from ceruloplasmin. These observations provide additional evidence that the 138K band represents the native ceruloplasmin molecule.

As stated earlier, PT only weakly interacted with the 50K ceruloplasmin fragment (one *N*-linked oligosaccharide chain) on western blots (figures 7, 8, 11, and 12). However, when the ability of the 50K ceruloplasmin fragment to inhibit the binding of PT to fetuin was examined using the fetuin-coated polystyrene tube assay, the 50K fragment strongly inhibited the binding of PT to fetuin (figure 14, table 2). As was previously mentioned, Glycophorin A (one *N*-linked oligosaccharide chain) could also inhibit the binding of PT to fetuin at μM concentrations in the fetuin-coated polystyrene tube assay (table 1; Tyrrell, G.J., *et al.*, 1989). However, when PT binding to Glycophorin A immobilized to nitrocellulose was examined, the PT also only weakly interacted with the glycoprotein (Witvliet,

M.H., *et al.*, 1989). The reason for this discrepancy was believed to be related to the valency of glycophorin A (and the 50K fragment) in solution. It was suggested that, in solution, the glycophorin A was able to form aggregates, and subsequently bind to PT through multivalent interactions. Analysis of the 50K ceruloplasmin fragment by Sephadex gel filtration (G-100) clearly demonstrated that the 50K fragment does aggregate when in solution, [the 50K peptide had an apparent molecular weight of greater than 158,000 (table 3)]. These results emphasize that multivalent interactions are important for PT binding, and also stress that the avidity of PT-glycoprotein interactions cannot be readily assessed when the glycopeptides are attached to a solid matrix such as nitrocellulose.

The 72K (67K) fragment was also shown to aggregate in solution, thus, when in solution, precise valency of the 72K (67K) fragment is unknown. Therefore it is not possible to comment on the optimum valency required for PT binding in solution. However, analysis of the binding activity 72K (67K) fragment by the western blotting procedure showed that for optimal PT binding, a valency of three is sufficient. This statement is based on the finding that on western blots, the 72K fragment binds to PT as well as the 138K fragment (4 oligosaccharide chains).

5.0 CONCLUSIONS

My initial studies of (native and R/A) ceruloplasmin-PT and ceruloplasmin-WGA binding interactions using the polystyrene tube binding assay, demonstrated high affinity lectin-glycoprotein interactions. As suggested previously by Tyrrell, *et al.*, (1989), although pertussis toxin and wheat germ agglutinin recognize common sugars in complex oligosaccharides, the two molecules may prefer different sugar sequences. Factors such as: the number of oligosaccharide units per glycoprotein (valency), amino acid linkage (*N*-linked *vs.* *O*-linked) of the oligosaccharide chains, sequence of the core sugars, terminal NeuAc linkage [(α-2,6)-linked vs. (α-2,3)-linked], geometric orientation, and whether or not the oligosaccharides are biantennary, triantennary, or tetraantennary structures, may play an important role in pertussis toxin binding. Pertussis toxin appears to bind to a variety of glycoproteins with varying oligosaccharide structures; however the avidity of the interaction appears to vary markedly. Though various possibilities for the differential binding have been suggested (Tyrrell, G.J., *et al.*, 1989), further studies to confirm their validity were warranted. Therefore, we continued to analyze PT-carbohydrate interactions in hopes of more precisely defining host cell receptors for PT.

A large body of evidence suggests a requirement for *N*-linked oligosaccharide chains (Armstrong, G.D., *et al.*, 1988; Brennan, M.M., *et al.*, 1988) with terminal sialic acid residues α(2-6)-linked to galactose (Armstrong, G.D., *et al.*, 1988; Brennan, M.M., *et al.*, 1988; Tyrrell, G.J., *et al.*, 1989; Heerze, L.D., and Armstrong, G.D., 1990). In addition, recent evidence implies that glycoproteins with solely biantennary oligosaccharide chains are capable of strongly interacting with PT (Heerze, L.D., and Armstrong, G.D. 1990). Evidence for multivalent interactions has also been postulated (Armstrong, G.D., *et al.*, 1988; Tyrrell, G.J., *et al.*, 1989).

Although the ceruloplasmin model does not allow all of the factors of PT binding to be assessed, it did accommodate many of them. The precise valency (number of oligosaccharide chains) required for optimal PT-glycopeptide interactions is unknown, however the results suggest that when the glycopeptide is attached to a solid matrix such as nitrocellulose, a valency of one may be inadequate. However, when the inhibiting glycopeptide is in solution, a valency of one may be sufficient, if the glycopeptides can interact with each other and increase their valency. These statements are based on the minimal PT-binding activity to the 50K ceruloplasmin fragment in western blot assay (figure 12), the ability of the 50K fragment to strongly inhibit the binding of PT to fetuin in the polystyrene tube assay (figure 14, table 2), and the

data that suggests that both glycophorin A (Furthmayr, H., *et al.*, 1975) and the ceruloplasmin 50K fragment (figure 15 and table 3) aggregate in solution.

It was also shown that in the western blot, a valency of three is sufficient for maximum binding: the 72K (67K) fragment was found to bind to PT as well as the native molecule (which contains four *N*-linked oligosaccharide chains). It was also shown that the 72K (67K) fragment aggregates in solution, and thus, when in solution, the precise valency of the 72K (67K) fragment is unknown. However, the western blot analysis of the PNGase F-treated ceruloplasmin indirectly addresses the valency issue. It was evident that incomplete removal of the oligosaccharide chains had occurred for some of the ceruloplasmin fragments (figure 11). Although the exact valency of these peptides is unknown, it is known that the valency is lower than three, because the partially deglycosylated peptides migrated farther on SDS-PAGE gels than the native peptides. This provides further evidence that a valency of less than three oligosaccharide chains does not allow for optimal PT-binding activity in western blot-type assays. Also, the deglycosylated ceruloplasmin had a greatly reduced activity in the polystyrene tube assay. Therefore, glycopeptides with valencies of less than three may have some PT-binding activity, but this activity is quite limited when the glycopeptide is attached to a solid matrix such as nitrocellulose.

The ceruloplasmin model also demonstrated the importance of the oligosaccharide portion of glycoproteins in PT-binding interactions. Previous studies (Armstrong, G.D., *et al.*, 1988; Brennan, M.M., *et al.*, 1988; Tyrrell, G.J., *et al.*, 1989) suggested but did not prove the importance of the *N*-linked oligosaccharide unit. In the present investigation, removal of the *N*-linked oligosaccharide chains of ceruloplasmin using PNGase F was found to abolish PT-binding activity.

Another important observation of this study was that reduction and alkylation of disulfide bonds of ceruloplasmin had no apparent affect on PT-binding activity in the polystyrene tube inhibition assay (table 1). In fact, reduction and alkylation appeared to slightly enhance the interaction of PT with ceruloplasmin (figure 13, and table 1). Further analysis of R/A ceruloplasmin in western blots confirmed this observation (figure 7, panel B). It was also demonstrated which of the ceruloplasmin peptides seen on SDS-PAGE gels were capable of binding to PT. In addition, separation of the individual ceruloplasmin bands by SDS-PAGE, and subsequent elution from the gels also did not alter ceruloplasmin's PT-binding activity (figures 12 and 14, and table 2), in the western blot and polystyrene tube inhibition assay.

Using the ceruloplasmin model, further information about the specific geometric orientation of the oligosaccharide chains could be determined. Because

boiling the ceruloplasmin samples prior to SDS-PAGE has linearized the ceruloplasmin structure (to a large degree), the western blot analysis has demonstrated that the linear secondary structure of ceruloplasmin confers optimal PT-binding activity, and perhaps only minimal additional tertiary structure is believed to be required. The primary amino acid sequence of ceruloplasmin allows for the correct geometric orientation of the *N*-linked oligosaccharide units to facilitate attachment of the PT or WGA. Because the distance between the oligosaccharide chains of ceruloplasmin is known, it could possibly be used as a model for approximating the distance between linkage sites of oligosacchaide chains to a peptide backbone. However, studies of this nature would require additional information from X-ray crystallography studies about the geometric orientation of the peptide backbone and oligosaccharide units. The ceruloplasmin model provides further insight into PT's lectin-like properties, and may help to establish the relationship between those receptors for PT on glycoproteins, and those receptors on human tissue cells.

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